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PRINCIPAL INVESTIGATOR: Ward Casscells, M.D.

CONTRACTING ORGANIZATION: University of Texas at Houston
Houston, Texas 77030

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The DREAMS basic science program complements the digital EMS program and has identical goals: faster diagnosis and treatment. We are studying how the lungs and liver synthesize its P450 defenses against toxins, so that we can accelerate that response and prevent chemical and biological injury. To speed diagnosis, we are developing fluorescent and luminescent markers for pathogens, cell types and cell injuries. We are developing heat imaging techniques, to triage victims and to diagnose injuries, including deep areas of hemorrhage, necrosis or inflammation.

We are applying new treatments for cardiac arrest and cardiac failure, to sustain patients until hospital arrival, including the use of a field-implantable cardiac assist pump, made biocompatible by genetically engineered cells.

To limit reperfusion injury, we are testing inhibitors of inflammation, oxidation and apoptosis. To speed healing, we are targeting macrophages and studying angiogenic growth factors. Many of these applications are supported by our new techniques that facilitate gene therapy. This is a broad and integrated program with both near-term and long-term benefits and a high likelihood that the basic research will spin off applications not yet foreseeable.

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W Rossell 4-14-95

PI - Signature Date

ANNUAL PROGRESS REPORT

PROJECT TITLE: "Disaster Relief and Emergency Medical Services Project (DREAMS): Clinical and Basic Science Projects"
THE UNIVERSITY OF TEXAS - HOUSTON HEALTH SCIENCE CENTER
PRINCIPAL INVESTIGATOR: Ward Casscells, M.D.
GRANT NO: DAMD17-98-1-8002
REPORTING PERIOD: 11/1/97- 10/31/98

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GRANT NO: DAMD17-98-1-8002
REPORTING PERIOD: 11/1/97- 10/31/98

EXECUTIVE SUMMARY OF THE DREAMS PROGRAM

PREVENTION.

A novel approach to protection against biological and chemical agents has been undertaken by Henry Strobel, Ph.D. He and his group are working to up-regulate the enzymes of the cytochrome P450 system in the lung. The many enzymes in this system have been extensively studied in the literature, but the detoxification functions of the lung are not well understood. Over this past year, they have characterized the regulation of one of these enzymes, 4F5, a prerequisite to any attempt to increase the expression of the gene. They have identified a nontraditional initiation site (CCAAT) as well as the SP1 binding site seen in some other P450 genes. They have identified retinoid and NF B binding sites, suggesting that 4F5 plays a role in the inflammatory response.

They have also identified some of the compounds that 4F5 inactivates, including the drug chlorpromazine. Also, 4F5 converts arachidonic acid to 20-hydroxyeicosatetraenoic acid, again suggesting a role in the inflammatory response.

An alternative to up-regulation by chemical agents is increasing the number of copies of the gene itself, which they have begun to do, using lipofectin and lipofectamine to express the gene for the green fluorescent protein in cultured macrophages. The expression in only one percent of cells is leading them to look for ways to increase this expression level prior to transecting the 4F5 gene.

They are also studying the expression pattern of 4F5 in lung tissue, a prerequisite to demonstrating its up-regulation.

DIAGNOSIS

It is well recognized that triage and early diagnosis of victims of wound and other emergency conditions are suboptimal. David Engler, Ph.D., is developing new fluorescent and luminescent imaging of molecules on the surface of cells. The goal is 1) to permit instantaneous diagnosis of chemicals and microbial pathogens, 2) to better diagnose wounds by determining the types of cells and their degree of activation and disease, and 3) to eventually guide surgical treatment and monitor response to medical treatments. To this end, he has shown that fluorescent and luminescent antibodies can be used to discriminate endothelial cells from smooth-muscle cells and to distinguish activated endothelial cells by their expression of adhesion molecules, cytokines and immunologic markers on their surfaces, in the living slate. He has shown that luminescent techniques enable some of these to be detected with the naked eye, which conceivably could permit surgery under low-light conditions, such as those on the battlefield.

Ward Casscells, M.D., has made an observation that may improve battlefield triage and prognosis: The best predictor of imminent death in patients with congestive heart failure was found to be a low core body temperature. The reasons for this are being explored, but this novel finding suggests that patients with hypothermia on the battlefield need urgent attention. It is not yet known whether there is a temperature that suggests futility of treatment, nor is it clear that these observations in patients with congestive heart failure apply to patients with trauma, but, interestingly, this novel finding was not a surprise to James H. Duke,

M.D., in whose clinical experience with trauma patients, the finding of hypothermia has been an adverse risk factor, though not yet published.

Dr. Casscells and Dr. Willerson have also found, in conjunction with Radiology colleagues, Larry Kramer, M.D., and Ponnada Kramer, M.D., that magnetic resonance imaging can be used to detect thermal heterogeneity. It remains to be seen whether thermal MR can be developed into a clinical tool for the detection of areas deep in the body that are warm (suggesting inflammation) or cool (suggesting ischemia) or cold (which might suggest tissue death).

TREATMENT

Cardiac Arrest. Trauma, toxic exposures, burns and other emergencies are often complicated by cardiac arrest, even in patients without known heart disease. A recent advance is the automatic external defibrillator, which has proven its utility in the hands of EMTs, firemen and policemen. Drs. Casscells and Duke are attempting to test the utility of automatic external defibrillators for the first time on a county-wide scale, in Harris County (the nation's "leading" county in industrial accidents). Considerable progress has been made in the deployment of automatic external defibrillators in the city and surrounding county and, data are accumulating about their effectiveness.

Cardiac Failure. Tissue survival after trauma or other emergencies requires sufficient hemoglobin, oxygen and cardiac output. The latter can be compromised blunt or penetrating chest injury, toxins, hypoxemia, excess catecholamines, conduction disturbances and other causes. O. Howard Frazier, M.D., and colleagues are pursuing a direct approach. Their goal is to determine the best of several commercially available small pumps that, in theory, can be inserted in the field. This project, a collaboration with Brook Army Medical Center, has begun with the development of a canine model.

A related effort has been that of Tim Scott-Burden, Ph.D., who is working to develop cellular linings for cardiac pumps for left ventricular assist devices and other cardiac pumps, such as the total artificial heart, and for arterial stents. The goal is to prevent the platelet attachment (which in the case of the left ventricular assist devices causes thrombocytopenia and hemorrhage), as well as thromboembolism. One problem is that endothelial cells are rather easily detached in response to high shear or turbulence. Dr. Scott-Burden has confirmed previous reports that ascorbic acid increases endothelial cell resistance to detachment. In addition, transfection with the tropoelastin gene maintains endothelial attachment. Nevertheless, vascular smooth-muscle cells form a more durable lining. Their problem is that they are not as capable of preventing thrombosis as endothelial cells. Therefore, he has engineered them to over-express the gene for inducible NO synthase and has found that this reduces platelet attachment. His eventual goal is to engineer smooth-muscle cells to enhance endothelial regeneration. To this end, he has transfected endothelial cells with the gene for vascular endothelial growth factor and has found that this enhances endothelial regrowth *in vitro*. Some other spinoffs of this work have been the discovery that smooth-muscle cells also respond to vascular endothelial growth factor when over-expressing the gene for fibroblast growth factor 2. He has also found that when endothelial cells are engineered to over-express VEGF, they increase their responsiveness to FGF2, without an increase in FGF receptors. The endothelial cells over-expressing FGF2 were found to be more responsive to interleukin 1 beta in terms of their production of nitric oxide.

Reperfusion/Resuscitation Injury. Richard W. Smalling, M.D., Ph.D., has found, in a canine model of coronary occlusion, that adenosine can mitigate reperfusion/resuscitation injury. He also has found that stretch of the myocardium increases the expression of adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1.

Apoptosis. The induction of apoptosis during reperfusion/resuscitation injury causes further injury. Inhibition of inappropriate apoptosis may thus be a mechanism of limiting reperfusion/resuscitation injury. An inhibitor of apoptosis has been discovered and named sentrin by Ed Yeh, M.D., Ph.D., who has cloned the gene and is studying its regulation and the mechanisms by which it regulates the apoptosis signal and cascade. Another finding that may be relevant is that of W. Casscells, M.D., and J.T. Willerson, M.D., who have found that gentle heating (e.g., to the 40 to 42° C. range) can selectively cause apoptosis of macrophages. Among the many genes they have found to be regulated by this kind of heat exposure are the FGF

receptors. Their up-regulation, together with that of the heat-shock genes, is likely to improve the survival of the parenchymal (noninflammatory cells). Thus, prior exposure to heat may have a double mechanism of improving the response to reperfusion/resuscitation injury.

Healing. Ed Yeh, M.D., and James T. Willerson, M.D., have found that macrophage targeting can be blocked by an antagonist of the E and L selectins. This suggests a number of candidate approaches to increase or decrease the inflammatory response.

The up-regulation of FGF receptors by heat, described above, suggests that properly dosed and properly timed heat exposure could be used to enhance angiogenesis.

Finally, Tim Scott-Burden, Ph.D., has discovered that the usual form of liposomal vector for gene transduction (lipofectin^{tm}) has the previously unsuspected effect of inducing nitric oxide production. This raises important questions about the thousands of gene transfer and gene therapy experiments that have been described to date. Furthermore, Dr. Scott-Burden has evaluated another commercially available transfection agent and has found that it inhibited production of NO.

Dr. Eric Boerwinkle has been studying genes involved in thrombosis, inflammation, cell proliferation and vascular tone, using two clinical endpoints that may serve as model for other condition, including traumatic wounds. He and his colleagues have found that elevated levels of intercellular adhesion molecule 1 and E-selectin predict the development of atherosclerotic disease. They have also found that genetic polymorphisms in the genes controlling the renin angiotensin system explain some of the variance in the development of hypertension and myocardial infarction.

Taken together, these data demonstrate progress toward the prevention, diagnosis and treatment of a variety of types of wounds. Moreover, many of the basic studies have implications in fields other than wound biology, including cancer, immunology and cardiovascular disease.

INDIVIDUAL PROJECT ANNUAL REPORT DETAILS

Project 1C: Automatic External Defibrillator Project
Principal Investigator: S. Ward Casscells, M.D.

This project has as its goal the reduction of deaths due to out-of-hospital sudden cardiac arrest. A major focus has been increasing the implementation of the automatic external defibrillator. The problem is that there are 350,000 sudden cardiac arrests each year in the United States. The vast majority occur outside the hospital, and, according to the latest figures, while most victims are taken to the hospital, only 5 percent are discharged alive. The problem is time. Brain cells begin to die after four minutes, and as every minute passes, the chance of death increases by 10 percent. The widespread adoption of cardiopulmonary resuscitation (CPR) has helped, but numerous studies have now shown that immediate defibrillation is preferable to CPR, since CPR typically only generates a blood pressure of 60 to 70 systolic and ventilation is often inadequate as well, resulting in inadequate delivery of inadequately oxygenated blood to the brain and other vital organs.

It has been estimated that immediate defibrillation would result in the salvage of an additional 100,000 lives a year, or 30 percent of all those afflicted. This goal has not been even close to being attainable, because defibrillators are costly (such that few institutions other than hospitals have them) and are too complicated for any but regular users to employ.

These problems have been addressed by the recent development by several companies of relatively inexpensive ($3,000 to $3,500) lightweight (2 to 3 pounds), easy-to-use, semiautomatic defibrillators. Some require only that the box be opened, at which point verbal instructions are given to attach electrode pads to the chest as shown in the diagram. After analyzing the rhythm, the voice then advises the rescuer to either perform CPR or to press the defibrillation button (after warning other bystanders to stand back and not contact the victim, so that they are not shocked). In practice, these devices have proven 98 percent accurate in the detection of ventricular fibrillation, and completely untrained users generally have been able to use them successfully. Though they are virtually foolproof, the number of devices sold and used has been relatively small. When DREAMS was conceived three years ago, our interest was to incorporate early defibrillation into a systematic emergency medical services program. We began by studying the barriers to early defibrillation and found that they were numerous. Few people were aware of the technological improvements, few realized the limitations of CPR, very few automatic external defibrillators were available, and few agencies (e.g., police, fire and even EMS) had taken an interest. The few companies that had taken an interest on behalf of their employees had been advised that they risked liability. In other words, it was unlikely they could be sued if someone died in the workplace, but the lawyers were concerned their companies could be sued for failed defibrillation or for late defibrillator that resulted in the salvage of a brain-damaged victim.

Our approach to this problem has been to begin with doctors and nurses, EMTs and paramedics. To this end, we have put on a series of educational programs throughout Houston for doctors, medical students, nurses, paramedics and EMTs. We have made progress on many fronts. For example, our advocacy (and that of others) has resulted in the American Heart Association making emergency treatment of cardiac arrest a top priority of the national organization. We also succeeded in making it a top priority of the Houston Affiliate (which has considerable independence with regard to the national organization). The

American Heart Association-Houston Affiliate now includes AED training in all basic CPR classes for health-care providers. The 1998-99 AHA-Houston program entitled "When Every Second Counts" has done a lot to educate the public as well, with numerous public demonstrations, corporate breakfasts, press conferences and resulting coverage in the Houston Chronicle, the Texas Medical Center News and numerous television stations and other media outlets, some of which are listed on the attached.
We also undertook a study of cardiac arrests taking place in Houston in 1997. As shown in the attached summary, some of the key findings were as follows:

The data collected by City of Houston EMS Medical Director David Persse, M.D., and medical student Laurie Fletcher revealed that of 1,343 primary cardiac arrests occurring in adults, for which the City of Houston Fire Department was called, 87 percent occurred at home or at a health-care facility, whereas 13 percent occurred at the workplace or in public places.

In 64 percent, the cardiac arrest was witnessed by a lay person and in 4 percent it was witnessed by an EMS personnel. Of those witnessed by a lay person, 58 percent received bystander CPR. In 64 percent of the cases, the first rhythm identified was ventricular fibrillation or ventricular tachycardia. Sixty-one percent of those arrests that did not occur in hospital or at home occurred in private businesses, 18 percent in public facilities and 21 percent on public streets. Of particular note is the fact that the City's major airports had the highest incidence of cardiac arrest. The highest frequency was in the 45- to 54-year-old age range. Interestingly, most of the calls came between noon and 6 p.m. After that, the most frequent time was between 6 a.m. and noon. The average time from 911 call to arrival of EMS personnel was 6.4 minutes. In 45 percent of cases, the return of a pulse was documented, but in only 15 percent of cases was the patient discharged alive from the hospital. Further details are provided in the attachment.

These data indicate that there is room for improvement and suggest that the widespread availability of automatic defibrillators would result in an enhanced rate of return of pulse and presumably of discharge from hospital without major brain damage.

We have made and distributed a video about automatic external defibrillators, a copy of which is included with the report.

To this end, we have increased our efforts and the progress include the following:

1. All City of Houston fire engines now carry AEDs (vs. 15 percent nationally).
2. All EMS vehicles (but not paramedic-manned vehicles) carry AEDs (vs. 50 percent nationally).
3. George Bush Intercontinental Airport, Hobby Airport, the Astrodome and the Memorial Park police cruisers are all now equipped with AEDs.

Corporate awareness has increased markedly, with defibrillators now becoming available on many airlines flying in and out of Houston, including American, Delta and Qantas. The number of corporations that have purchased AEDs is 45 and increasing steadily. Because of concerns about liability, we have persuaded legislators to sponsor bills to provide some protection from liability to those corporations that purchase AEDs, provided they maintain them properly. Expenses incurred over the 12 months were 184,333, mostly for the educator/data collector and nurse-practitioner, and for salary support for

administrator Johna Kincaid, a re-budgeting approved by USAMRMC in January 1998.

The goals for FY '99 will include continued efforts in all of the areas described above. In particular, we hope to persuade the City to spend the $7 million necessary to put an AED in every police cruiser, and we hope to have an Houston Chronicle editorial in addition to continued television features on this topic. As the dissemination and use of AEDs continue to increase, we will be in a position to evaluate their effectiveness. We are documenting the number of defibrillators purchased and their location, the number of times they are used, the percent that are successful (the main metric being discharge alive from the hospital). A secondary endpoint is return to work. The controls will be those patients resuscitated by defibrillation by EMTs or paramedics. A third endpoint is the average time elapsed between 911 call and deployment of a defibrillator by EMS or bystander. We will also note whether or not the bystander has had prior training. Also, we will be recording building-specific data, comparing successful resuscitation rates in building with AEDs vs. buildings without AEDs.

Project 1D "Effect of Inhaled NO on Oxygen Saturation in Victims of Airway Burn Injury"

Principal Investigator: Robert Lodato, M.D.

Project completed several iterations of CPHS protocol review and ultimately was denied approval. Project has been terminated.

Project 1E: "Effect of Liposomal PGE1 and Selectin Antagonists in Acute Myocardial Infarction"

Principal Investigator: Richard W. Smalling, M.D.

Project has been through several iterations of CPHS protocol review. Administrative personnel have submitted to U.S. Army IRB offices a request for clarification of the latest instructions. Clarification from U.S. Army is pending and we look forward to complying with IRB requirements and achieving CPHS approval.

Project 1F. Fibroblast Growth Factor (FGF-2) to Enhance Angiogenesis and Healing.

This clinical trial designed and approved here at the University of Texas-Houston Medical School and Hermann Hospital and approved by the Army IRB accounted for almost all of the $41,352 spent during the year. The project is now on hold by the Food and Drug Administration because of concerns about proteinuria experienced by patients in a different protocol (claudication patients given multiple doses of FGF-2).

The pause in the clinical trial has not prevented useful work in this area, however, as we have found that fever up-regulates FGF receptors, as described for Project 2A (below). This has been confirmed in living human carotid artery specimens immediately after surgical removal (e.g., in the living state) and has led us to hypothesize that the FGF system is an acute stress response system and not only a wound hormone and angiogenic factor. In 1999, therefore, we will use the balance of these funds to explore the role of FGF and FGF receptors in heat therapy, as further described under Project 2C (below).

Project Number: 1G

Investigator Name: Alan S. Tonnesen, MD

1. Obtain approval from UTHHSC Committee for Protection of Human Subjects (CPHS):
1.1. Final approval for this portion of the project was received from the UTHHSC Committee for Protection of Human Subjects
2. Hire systems analyst:
2.1. We have found an Emergency Medicine physician who has extensive computer skill who will be ideally suited for this project and is very interested in the concept. He will be interviewed and if suitable, an offer will be made.
2.2. Goals for 1999
2.2.l. Hire analyst
3. Purchase and set up server.
3.1. We have not purchased the server, pending hiring of a systems analyst. The requirements for the server are outlined.
3.2. Goals for 1999
3.2.1. Purchase server and required software
3.2.2. Set up NT server
3.2.3. Set up Web server for intranet
3.2.4. Set up database for incoming monitored data
3.2.5. Install Electronic Medical Record (EMR) on server, workstation and medical Control work station
3.2.6. Train personnel in use of EMR
4. Convert current EMS/ Life Flight protocols to web based format and incorporate into the medical control process.
4.1. Medical and administrative protocols include:
4.1.1. Management of tachycardia
4.1.1.1. The third iteration was completed.
4.1.1.2. Goals for 1999:
4.1.1.2.1. Test tachycardia algorithm for feasibility in classroom setting
4.1.1.2.2. After feasibility of tachycardia algorithm is demonstrated, apply for CPHS approval to institute clinical trial
4.1.2. Management of acute pulmonary edema, hypotension and shock
4.1.2.1. These protocols have not been implemented.
4.1.2.2. Goal for 1999
4.1.2.2.1 Flow chart algorithms for acute pulmonary edema and shock
4.1.2.2.2. Test feasibility for field testing of algorithms for acute pulmonary edema and shock
4.1.2.2.3. Apply for CPHS approval and test in field setting of algorithms for acute pulmonary edema and shock
4.1.3.Facility diversion guidelines
4.1.3.1. The facility diversion guidelines have been extensively re-written, with the exception of seamless inclusion of helicopter transport.
4.1.3.2. Goal for 1999
4.1.3.2.1. Develop a comprehensive algorithm for facility diversion/ transport guideline

5. Goals for 1999
5.1. Hire analyst
5.2. Purchase server and required software
5.3. Set up NT server
5.4. Set up Web server for intranet
5.5. Set up database for incoming monitored data
5.6. Install Electronic Medical Record (EMR) on server, workstation and Medical Control work station
5.7. Train personnel in use of EMR
5.8. Test tachycardia algorithm for feasibility in classroom setting
5.9. After feasibility of tachycardia algorithm is demonstrated, apply for CPHS approval to institute clinical trial
5.1.0. Flow chart algorithms for acute pulmonary edema and shock
5.1.1. Test feasibility for field testing of algorithms for acute pulmonary edema and shock
5.1.2. Apply for CPHS approval and test in field setting of algorithms for acute pulmonary edema and shock Develop a comprehensive algorithm for facility diversion/ transport guideline
5.13. Develop a comprehensive algorithm for facility diversion/ transport guideline

Project 2A. Evaluation of Basis Fibroblast Growth Factor (FGF-2) Injections to Maintain Tissue Viability in Rats with Hemorrhagic Shock.

FGF has caused hypotension (as much as 40 percent) in the rats, and of course this is undesirable in shock and may offset the beneficial effects of FGF (prevention of premature apoptosis). This, together with the halt called by the Food and Drug Administration because of unexpected proteinuria in one of the FGF clinical trials, has prompted us to develop an alternative strategy for prevention of tissue death and hemorrhagic shock. Specifically, we have found that the FGF receptors are up-regulated fivefold by gentle heating (41o C X 15 min.). This immediately suggests an alternative to FGF injections for activation of FGF receptors. The potential therapeutic benefits include delay or avoidance of excess tissue apoptosis in the setting of shock, trauma, hypoxia or toxin exposure, promotion of angiogenesis and promotion of wound healing. The experimental and clinical protocols for therapeutic pre-exposure to heat are described in more detail in Project 2C (below).

Expenditures as of November 1, 1998, have been $16,784.

Project Number: 2-B Mechanisms of Infarct Salvage With Selective Adenosine Infusion In Regional Myocardial Ischemia
Investigator Name: Richard W. Smalling, M.D., Ph.D.

Work on DREAMS Project 2B, "Mechanisms of Infarct Salvage with Selective Adenosine infusion in Regional Myocardial Ischemia", began September 1998 after delays in project selection and the approval process. Under the direction of Richard Smalling MD, PhD., a team consisting of Michael Rihner, MD cardiology fellow, Hela Achour MD volunteer and technicians James Amirian, Patty Felli and Camell Parks have made significant progress in our first quarter of work. The 0.14 infusion guide wires (180 cm and 350 cm wire lengths, 2 cm infusion length) were obtained from the TherOx Company in Costa Mesa, California.

Due to problems encountered in the placement of the 7 F left Judkins guiding catheters (FL3) into the left anterior descending artery via the femoral 9F sheath in the first four experiments a slight change in protocol was initiated. After switching to an Amplatz (7F ARI) guiding catheter via the left internal carotid artery to the LAD , less complications with shorter catheterization times have occurred. The TherOx infusion wire have been reliable and durable during the catheterization and infusion process.

Currently to date a total of 17 experiments have been performed. Of these, 10 experiments have gone to completion and the hearts/data are in the long process of analysis (6 canines in the 'Adenosine infused group and 4 canines in the 'Control' group). Five animals suffered ventricular arrest during the occlusion of the LAD with revival efforts unsuccessful . There were two animals that were rejected after instrumentation and surgical complications.

The mortality rate is higher than expected for this protocol. The main problem was inferior heart vascular structure leading to weaker hearts that could not endure the infarction procedure. These animals arrested shortly after occlusion (within 1 5 minutes). An additional 1 0 canines will be requested from the UTH-HSC Animal Welfare Committee, however, not all these animals may be needed to complete the project. A minimum total of seven animals per group (Adenosine and Controls) will be needed for accurate statistical comparison in the final groups. Some of the completed experiments may be rejected if they do not meet standard criteria involving heart collateralization with pending radioactive microsphere determination of the regional myocardial blood flow. Sampling of each animal for RMBF data using radioactive gamma emitters 2S markers at baseline. IO minute occlusion of the LAD, 1 0 minute reperfusion , and 4 hour reperfusion during the experiment are currently being counted in a LKB CompuServe Gamma Counter. Hemodynamic and regional myocardial functioning data analysis is also pending and will also be taken in account for determination of the final animals used. It Is too early to present data in this first quarter report.

Planimetry of traced heart slices on acetate overlays will determine the size of the infarct percent of the zone at risk (ischemic area). The excess hearts were photographed after slicing and TTC staining for permanent record on a color contact sheet. Liquid nitrogen flash frozen tissue samples for a myeloperoxidase enzyme assay (WBC infiltration) were obtained and the assays will be performed in the next quarter. Immuno-histochemistry ICAM and P-Selecton expression staining tissue samples were preserved In OCT embedding compound. These samples include tissue in the nonischemic myocardium and ischemic myocardium (infarct and zone at risk areas) of the left ventricle. In addition, regional tissue samples have been preserved in 10% formalin and 3% glutaraldehyde. The pathology reports will be made by Dr. Margaret Ethonam of the UT Medical School Department of Pathology in the next two quarters. We hope to complete the remaining experiments in the next couple months, further analyze the experiments. present some data and determine which animals will be used In the final data for statistical comparison.

Project 2C. Infrared Detection of Inflammation.

This project is now broader than the original title suggests because it has not only proven successful in its original aims but has led to a number of spinoff projects that in fact have engulfed Projects 1F and 2A. Infrared is proving to be a very promising means of imaging inflammation and has also led to develop noninvasive methods of imaging heat, including thermal magnetic resonance imaging. The preliminary data described below suggest that we will not only have clinically useful heat-detecting catheters (infrared, thermistors and thermocouples), but thermal magnetic resonance imaging (or even thermal ultrasound and thermal microwave imaging) may prove useful in detecting inflammation, infection and tumors. We also believe that the detection of cooler areas will be a sign of necrosis. Needless to say, there is an enormous need for a diagnostic technique that will identify nonviable tissue in deep locations, such as those produced by blunt trauma or regional ischemia (e.g., due to hemorrhage). The project is described more extensively below, in 2 sections: A. Regulation of Inflammation by Heat, B. Thermal Imaging and Control of Inflammation. For each project, the model we are using is the inflammation of atherosclerotic plaques.

Expenditures as of November 1, 1998, were $248,469.

Project 2C
A. Regulation of Inflammation by Heat

PROJECT GOALS:

1) Determine whether heat regulates plaque inflammation and, if so, its mechanisms.
Hypotheses:
   a) In plaque macrophages and T-cells, heat in the fever range (39-41°C) down-regulates pro-inflammatory genes(e.g.IL1,IL2, MIP, IL6, IL8, TNFα), without causing apoptosis, and increases expression of anti-inflammatory genes (IL10, IL13, PPARα and PPARγ)
   b) Heat (≥40°C) inhibits macrophage DNA synthesis, generation of NO, ingestion of foreign bodies, NADPH oxidase activity, activation of NFkB, and secretion of matrix metalloproteases 1,3, and 9, thus constituting a negative loop feedback mechanism.
   c) Heat shock factor 1 is activated and one or more heat shock proteins are expressed in response to physiologic increases in plaque temperature (39-41°C), especially in conjunction with acidosis, hypoxia, oxidative stress or activation of PPARα and PPARγ by eicosanoids or drugs (eg salicylate, fenofibrate, or troglitazone).
   d) Inhibition of macrophages by heat is mediated in part by activation of heat shock factor 1 and induction of heat shock proteins (27, 32, 60, 70, 90, and/or 110); these responses protect macrophages against premature apoptosis.
   e) More severe (≥41°C), abrupt or prolonged heat causes plaque macrophages to undergo apoptosis, while endothelial and smooth muscle cells are more resistant
   f) The unusual sensitivity of plaque macrophages to thermal apoptosis at ≥ 41°C is due to multiple mechanisms (described later), and serves to prevent excess inflammation and injury to adjacent cells.

2) Determine the natural history of the hot plaque.

3) Determine whether heat therapy can prevent plaque rupture and/or erosion.

INTRODUCTION:
Despite the declining age-specific mortality of coronary atherosclerosis, many people who feel well and have no known cardiovascular disease continue to die suddenly of a first myocardial infarction or cardiac arrest. An estimated 35% had neither symptoms nor a diagnosis of coronary artery disease.[1, 2-4] The unpredictable nature of myocardial infarction, stroke and sudden cardiac arrest-which impedes prognosis and treatment-is in part due to the fact that rupture and/or thrombosis of an atherosclerotic plaque - the immediate cause of most myocardial infarctions and strokes is itself not predictable.[1-6]

Autopsy studies have demonstrated that the plaques that rupture are typically inflamed and have a thin fibrous cap[7]. The thinness of the cap has been attributed to a paucity of smooth muscle cells (SMC)—which produced the collagen—perhaps due to apoptosis.[8] Active digestion of the plaque by enzymes released from macrophages has also been demonstrated.[6]

We discovered that inflamed plaques give off more heat than non-inflamed plaques[9]. Inflammation has long been characterized by heat (*calor, tumor, rubor* and *dolor* being the classic features of inflammation), but local heat had not previously been described in atherosclerosis.

We reasoned that the surface temperature of the plaque's fibrous cap would be proportional to the density and metabolic activity of the inflammatory cells and to their proximity to the surface.

(List of abbreviations- please see appendix)

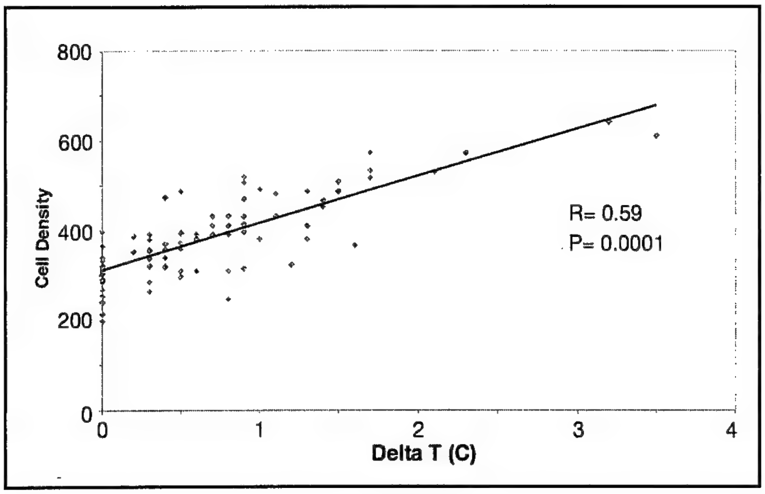


Figure 1. Graph demonstrating relationship of lumen temperature to underlying cell density in living human carotid atherectomy specimens. As described in Methods, temperatures of specimens were measured using a 27-gauge thermistor. The measurements were grouped into four, five or six isothermic zones. For a given patient, the median, coolest, and warmest zones are plotted in relative degrees C.

We studied 61 atherosclerotic plaques removed from 61 patients at the time of carotid endarterectomy performed for symptoms or for severe angiographic stenosis. Using a 24-gauge needle thermistor in a 37°C chamber, the specimens were examined at 2-mm intervals, yielding approximately 30 measurements per plaque. This established the background temperature. Typically, 6-7 zones per plaque were warmer or cooler than background by more than 0.2°C.

Some plaques were photographed immediately after removal from the 37°C incubator using a Santa Barbara Focalplane (Lockheed Martin, Santa Barbara, CA) Infra-red (IR) camera (Model IC 11000) which has a thermal resolution of 0.015°C. For each plaque, the background temperature and that of the warmest and coolest zones were marked with colorfast dye of different colors for subsequent localization on tissue sections. The tissues were then fixed in 10% formalin and processed for routine histology followed by staining with haematoxylon and eosin or Masson's trichrome or immunostaining for macrophages with the HAM-56 and KP1 (CD68) antibodies from Dako. Cell density was measured in a 200x400-micron (depth x width) region beneath the marked regions, using the program NIH Image.

In another series of experiments, Watanabe hypercholesterolemic rabbits were anesthetized and the aorta was removed, rinsed and studied by needle thermometry, routine photography, and infrared photography as described above.

Thermal heterogeneity of living human atherosclerotic plaques

As shown in Figure 1, the temperature of the lumen surface in living human carotid endarterectomy specimens varied by as much as 3.5°C within the same specimen. The reproducibility of the temperature measurements at these locations was within 0.2°C. All of the specimens demonstrated differences of 0.3°C. or more between their warmest and coolest measured locations. (Mean ± SD: 1.9±0.6°C).

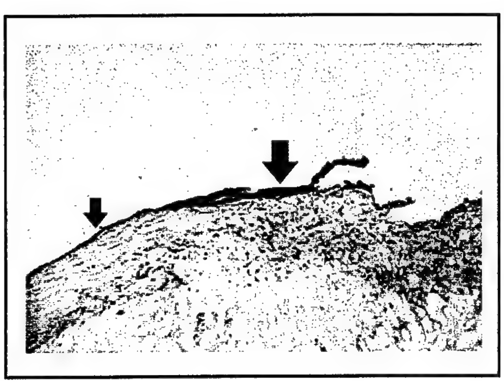


Figure 2. Photomicrographs of representative histology of cool region (small arrow) adjacent to a region of 0.6°C warmer in a living human carotid atherosclerotic plaque (marked in vivo by dye, and on the photo by a large arrow). The cap beneath the warm region is inflamed.

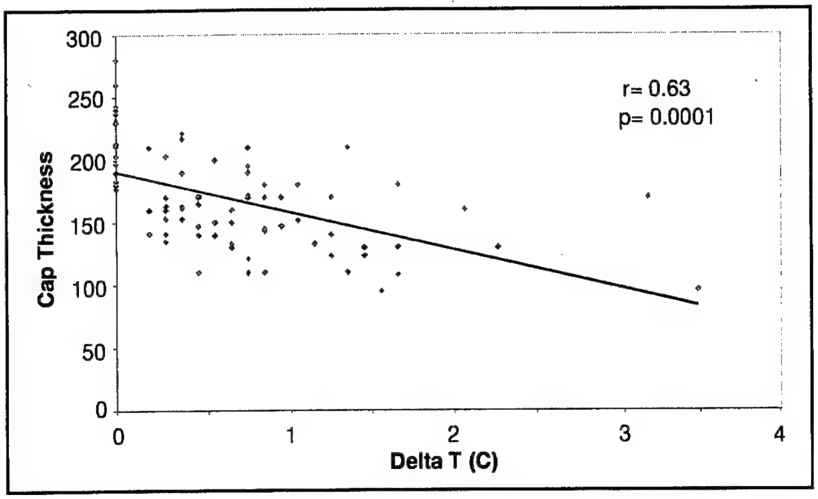


Figure 3. Inverse relationship of lumen temperature to the depth of the underlying cells in the plaque.

Histology of hot plaques

Figure 2 illustrates the histology of region 2.5mm apart, with the inflamed fibrous cap being 0.6°C warmer under the adjacent acellular region. As shown in Figure 3, the deeper the clusters of cells, the lower the temperature at the surface. Nevertheless, there was a statistically significant inverse relationship between cell depth (essentially, cap thickness) and surface temperature. The equations, $1/(\text{distance})^2$ and $1/(\text{distance})^3$ yielded no higher a correlation coefficient than 1/(distance), presumably reflecting the fact that most of the cells were not clustered in a point source of heat. In a subsequent series of plaques, we found a close correlation of heat with the density of monocyte-macrophages as shown in Figures 4 and 5. There was a negative correlation ($r = -0.4$) of heat with smooth muscle cell density. This paradox may be explained by the negative relationship between macrophage and SMC density ($r = -0.72$), perhaps due to induction of apoptosis in SMC by cytokines and NO from macrophages. We infer that macrophages generate much more heat than SMC.

Little or no Contribution of chlamydia pneumoniae to production of heat in the plaque cells

35 plaques analyzed in conjunction with Professor Kuo and Dr. Thomas Grayston in Seattle indicate no correlation of the presence of Chlamydia pneumoniae with areas of heat production (data not shown).

Relationship of angiogenesis and macrophage density to heat

To determine if heat is merely a function of vascularity, e.g. heat brought from the body core to the plaque, we graphed (Figure 6) lumen temperature against density of underlying capillaries and venules. In fact, there was a weak negative correlation: indicating that vascularity is not the sole cause of thermal hetero-

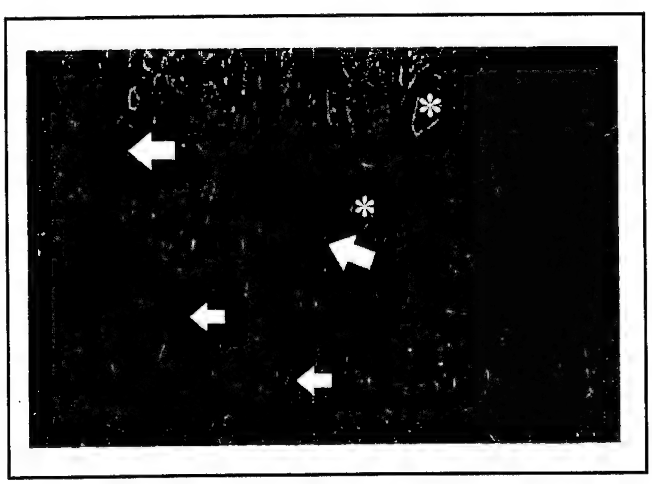


Figure 4. Photomicrograph of atherosclerotic plaque showing predominantly macrophages (large arrow), as indicated by immunoreactivity (dark brown) with HAM-56 antibody. Some cells with the appearance of smooth muscle cells are lightly stained (non-apoptotic) and marked with small arrows. Asterisks denote venules; most of their endothelial and smooth muscle cells are not undergoing apoptosis.

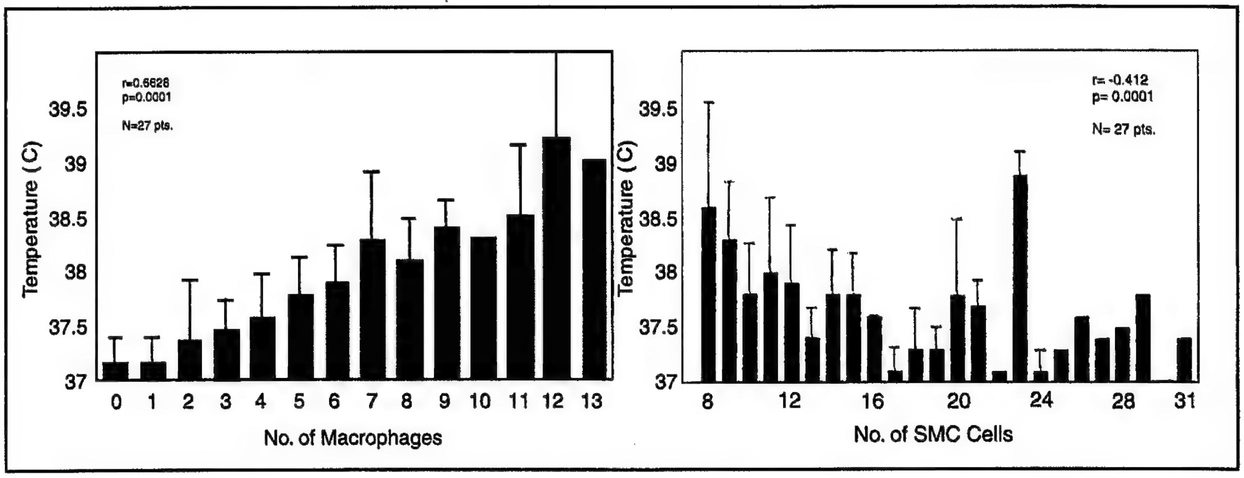


Figure 5. Graphs showing the dependence of temperature on the number of macrophages and smooth muscle cells. In 27 freshly harvested (living) carotid endarterectomy specimens from 27 patients, temperature was measured in a 37° incubator using a needle thermistor and colorfast dyes to relate the temperature to the underlying density of cells. Macrophages and smooth muscle cells were identified by their typical morphology (correlated in the first few specimens by immunocytochemistry with HAM-56 and CD-28 antibodies, and by electron microscopy).

geneity. In the intact plaque, blood flow may increase regional temperature or decrease it (by carrying heat away from a previously non-vascularized region). Therefore, we determined the temperature heterogeneity inside the intact aorta of the Watanabe rabbit, using a thermistor catheter (up to 1.5°C, data not shown) and IR thermography for the exterior of aorta of another Watanabe rabbit (Figure 7), despite the presence of normal blood flow. These data, together with the lack of correlation of heat with color, as shown below, suggest that 1) thermal heterogeneity of plaque is not just due to angiogenesis and/or vasodilation, and 2) thermal heterogeneity can be detected in vivo. Further evidence on this point is found below.

Poor correlation of heat with color

A photograph of a plaque with considerable thermal heterogeneity is shown in Figure 8. Note that the color of the lumen surface is a poor indicator of the temperature. This was true for the other plaques also, as indicated in Figure 9. Why should classic signs of inflammation – heat and redness - not co-localize? We hypothesize that red color may not discriminate fresh vs. old hemorrhage. Yellow may reflect cholesterol or esterified cholesterol, with varying amounts of carotene pigments. White may indicate collagen, which is not metabolically active, or platelets, which emit heat upon activation. Still another possibility is that surface color may mask deeper colors, while heat – being of longer wavelength – is less attenuated. The key inference is that direct vision (or angioscopy) can not provide clues for localizing macrophages; hence the need for a thermal device or alternative method.

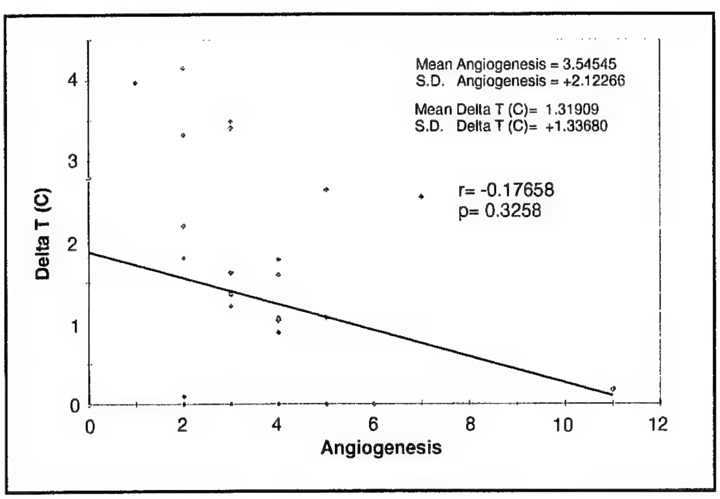


Figure 6. Graph depicting a weak inverse relationship of relative lumen temperature (recorded in human carotid atherosclerotic specimens immediately after removal by carotid endarterectomy) with underlying density of microvessels (capillaries and venules).

The potential of infrared detection

The detection of thermal heterogeneity by a thermistor suggested the potential of an infrared imaging catheter. We began by correlating mercury bulb thermometry with infrared thermometry, using heated beakers of water, which yielded a correlation co-efficient of r = 0.998

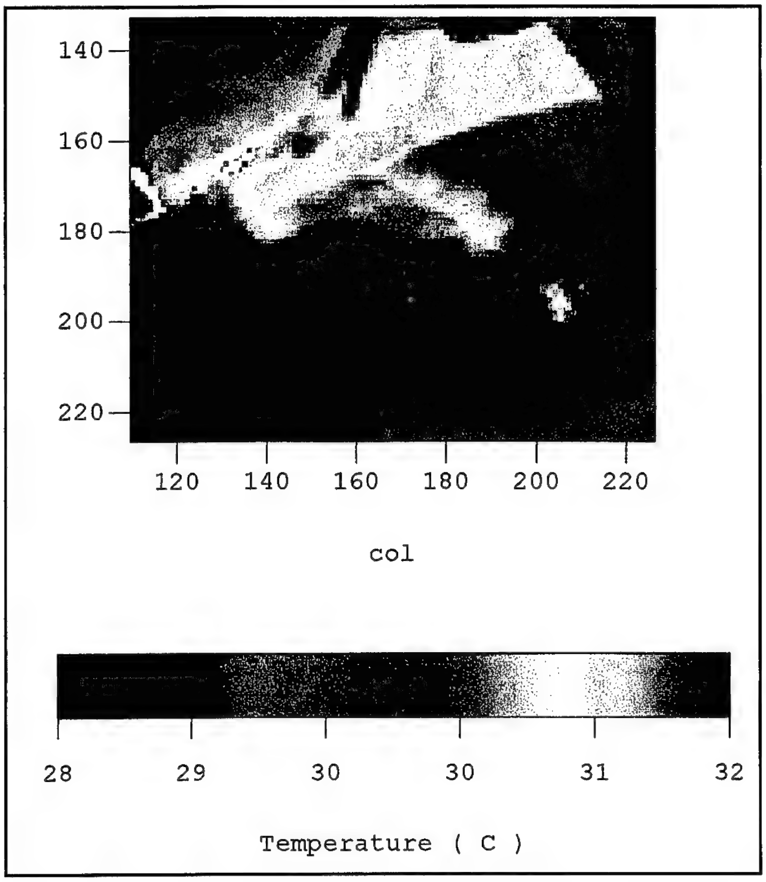


Figure 7. Infra-red photograph showing in-vivo temperature heterogeneity (arrows) in the thoracic aorta of Watanable hypercholesterolemic rabbit.

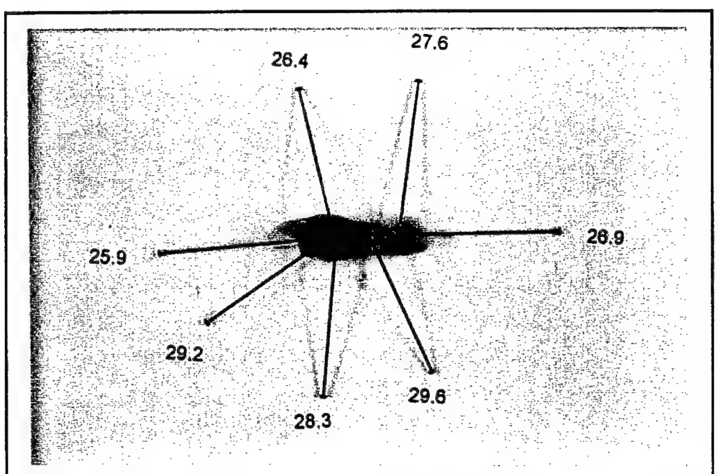


Figure 8. Photomicrograph of lumen of a fresh carotid endarterectomy specimen, with pins denoting the temperatures in a room at 25°C. Regions of similar gross appearance have different temperatures, and there is no obvious relationship of color to temperature.

(not shown). For wavelengths of 6-11μm, the emissivity of tissue is essentially that of water, but we verified this by correlating the needle thermistor measurements with the infrared thermographic camera in living human atherosclerotic plaque, as depicted in Figure 10. ($r = 0.988$)

We next related temperature to cell density using an infrared camera, as shown in Figure 11 ($r = 0.797$, ($p = 0.001$). Again there was an inverse relationship with the depth of the cells, as shown in Figure 12 ($r = -0.783$, $p = 0.0001$). Figure 13 shows an example of an infrared photograph taken of the luminal surface of a plaque immediately after removal in the operating room. Excellent spatial and thermal resolution is demonstrated.

In hemodialysis patients with arterio-venous (AV) grafts, the incidence of stenosis - attributable to inflammation, thrombosis and fibrosis - in the first postoperative year is 50-60 %.[10-12] We found the grafts to be superficial enough that their heat can be detected by infrared camera. In 25 images obtained from 6 patients undergoing hemodialysis, we also found considerable temperature heterogeneity (Figure 14). Interestingly, the mean temperature on the arterial side of the graft was 34.26°C± 0.93 vs. 35.03°C±0.62 on the venous side ($p<0.0001$), perhaps reflecting inflammation and/or cell proliferation, which typically are pronounced at the venous anastomosis. Analysis of the first 19 patients (mean 46 years old) showed, as expected, that graft flow was inversely related to graft age and patient age. The surprising finding was that flow was not proportional to the heat at the venous end, as might be expected since heat causes vasodilation and vasoconstriction reduces surface heat. Instead, as shown in figure 15, graft heat and flow were inversely related, consistent with the hypothesis that the inflammation and cell proliferation are generating heat and creating a flow-limiting neointima.

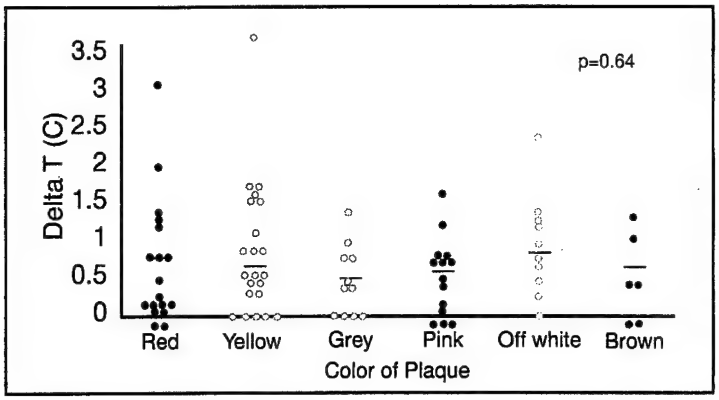


Figure 9. Graph showing poor relationship of regional color differences of the lumen surfaces to regional temperature, as assessed in freshly harvested, living (unfixed) specimens of human carotid atherosclerosis obtained at endarterectomy.

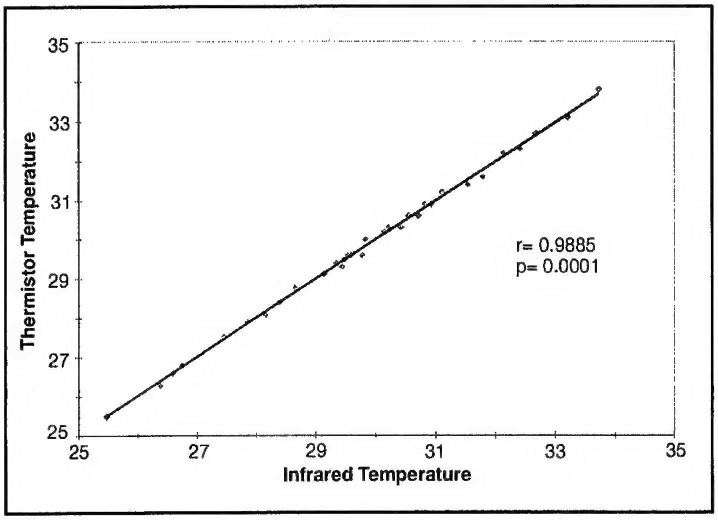


Figure 10. Graph demonstrating excellent correlation between thermistor temperature measurements of the lumen, human carotid plaque specimens, compared with measurements made with an infrared camera.

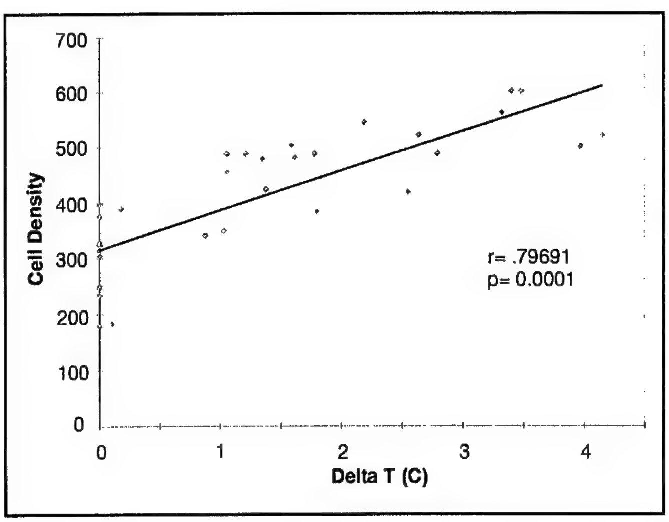


Figure 11. Relationship of lumen temperature (recorded with an infrared camera) to density of the underlying cells in 11 plaques. See text for details.

Similar heterogeneity was found in the Watanabe hypercholesterolemic rabbit carotid artery and aorta (not shown). Moreover, our veterinary surgeon Janice McNatt has developed a new model suitable for local heat measurements and interventions: by feeding these rabbits cholesterol, she created visible, palpably warm, atherosclerotic lesions that extended well along the central ear artery. (Figure 16) This model and the AV grafts have established 1) that thermal heterogeneity is detectable in the flowing artery, and 2) new models for following the natural history of the hot plaque.

The potential of heat therapy to induce apoptosis

Apoptosis is observed in atherosclerotic plaques.[10-16] Because apoptosis is a physiologic, non-inflammatory way of eliminating cells, it is attractive as a potential anti-inflammatory treatment. However, as described below, macrophage apoptosis has been little studied. Of the many physical and biochemical means of inducing apoptosis, we chose heating, reasoning that if an infrared catheter or other heat-localizing catheter could be developed, it would be relatively simple to reverse the direction of heat in order to apply heat to that specific lesion, without having to move the catheter.

To investigate this, we studied living human and rabbit specimens in an incubator immediately after removal. The specimen was divided, and one-half was left in the 37°C incubator while the other was placed for 15 minutes in an incubator preheated to 42°C. After a subsequent six hours in a 37°C incubator, there was a much greater increase in the percentage of macrophages undergoing apoptosis compared to SMC, in the specimen that had been briefly heated, as shown in Figure 17 and as graphed in Figure 18, as confirmed by the electron microscopy of macrophages. (Figure 19).

Heating causes cooling to below baseline

We noted that even gentle heating—to the range of 39-40°C for 15- minutes which causes little apoptosis—results in a subsequent cooling of the plaque below baseline, as shown in

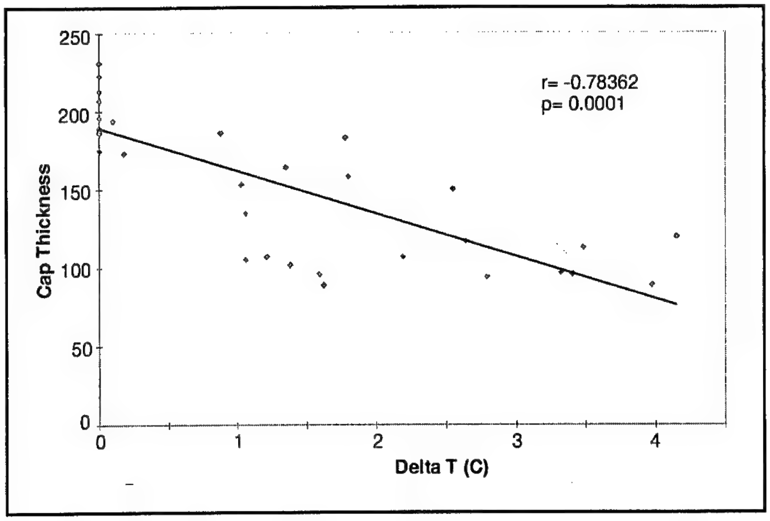


Figure 12. Graph depicting inverse relationship of lumen temperature (infrared) to depth of cells. Whether the cap was acellular or infiltrated, or its surface endothelialized or denuded, the distance from the lumen to the center of the cluster of underlying cells - of any morphology - was measured.

Figure 20. The effect was as large as that produced by 1 mg/ml indomethacin (though indomethacin acted gradually over 5 h whereas heat-induced cooling was fully apparent at 2 hours, suggesting different mechanisms). Moreover, 44°C caused no more fall in temperature than did 39-41°C (not shown) suggesting that apoptosis is not the major cause of post-heat cooling. The warmer areas (≥ 37.5°C.) cooled more than regions of normal temperatures (≤ 37.4°C). Together with our histologic evidence that macrophages are the main cellular component of the hot areas, this suggested the hypothesis that heat down-regulates the metabolic activity of macrophages, leading to the experiments described next.

Predicted Effects of Heat on Macrophages in Atherosclerotic Plaques

The predicted effects of heat on macrophages in atherosclerotic plaques is shown in Schematic 1.

Gene expression after gentle heating *in vitro*

Table 1 (please see appendix) shows the effects of 41°C on gene expression in cultured HL-60 macrophages. Note the 3-4 fold up-regulation of the stress-response (heat shock) genes and the down-regulation of most other genes, with the exception of the apoptosis genes, which show no significant trend (in three experiments). Especially striking is the 60-70% down regulation of the transcripts for IL-1β, IL-8, thymosin beta 10 and MCP-1. Less suppression (30-40%) was found for some of the cytokine receptors and integrins. Glutathione peroxidase transcripts were decreased by 80%. In another experiment (not shown) we found that this occurred within one hour following a 15-minute heating period. There was also an increase in tran-

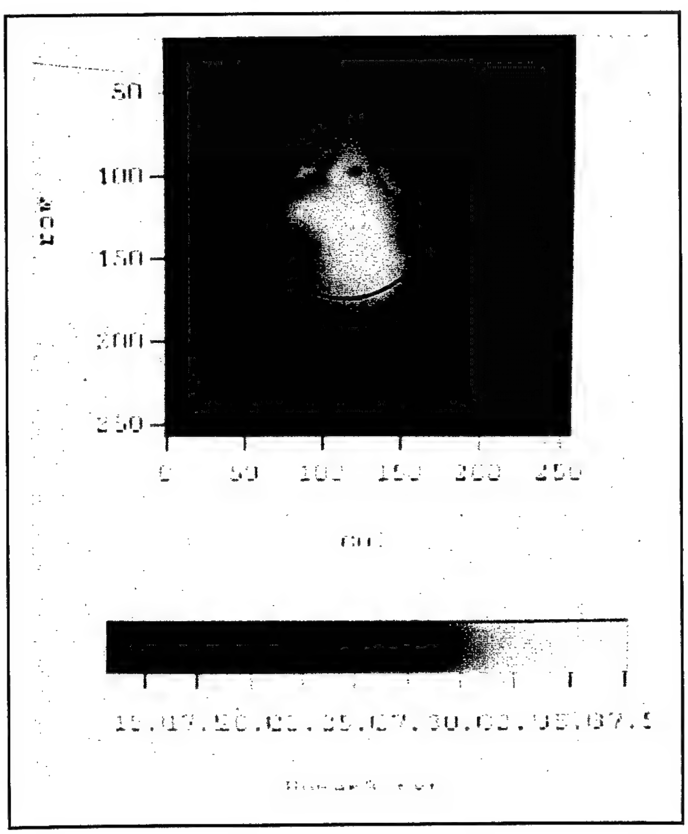


Figure 13. Ex-vivo infrared photograph taken in a 23°C room of living atherosclerotic plaque removed by carotid endarterectomy. The underlying scale reflects the calibration performed both electronically (by the "black body") and by correlation with beakers of water equilibrated at various temperatures measured by a mercury thermometer. Note the warm (25°C) central zone with a small red island (23°C) a few mm away.

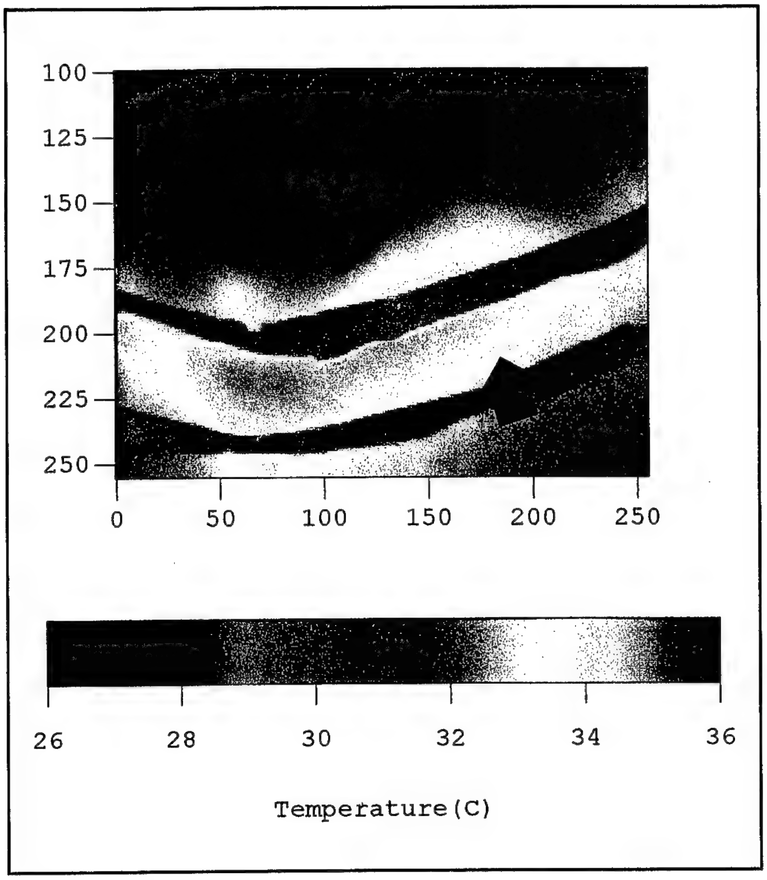


Figure 14. Infra-red photo of the prosthetic AV hemodialysis graft of a patient with renal failure. This graft, which had good flow and no palpable lesions or palpable temperature heterogeneity, revealed fine temperature heterogeneity by infra-red (arrows). This may represent another method for non-invasive detection and quantification of plaque temperature heterogeneity, and for following its natural course and its outcome after intervention.

scripts for IL-10. Combining these data with previous reports on other cell types, several mechanisms are thus suggested whereby heat may decrease macrophage activity (short of inducing thermal apoptosis) including 1) down-regulation of TNFγ, IL-1, IL-8 and MCP1 and up-regulation of anti-inflammatory IL10, 2) decreased NADPH oxidase activity[17], 3) heat-induced increase in PGE-1 synthesis[18], 4) an increase in superoxide anion[19], and 5) activation of HSF 1, which can inhibit activation of NFκB[20]. The decreased expression of inducible NO synthase would, according to the literature, be expected to act in the opposite direction, to increase macrophage activity.[21]

Discussion:

Our results demonstrate that living human and rabbit atherosclerotic plaques exhibit thermal heterogeneity on their lumen surface, and this relates well to the depth of underlying cells, most of which are macrophages. It is likely that lesions with inflammatory cells on the surface are dangerous because of the thrombotic tendency of these cells and the lack of antithrombotic and vasorelaxing endothelium. It is also likely that thin caps with inflammatory cells in or beneath the caps are at risk of rupture. However, we cannot yet conclude that a hot plaque goes on to rup-

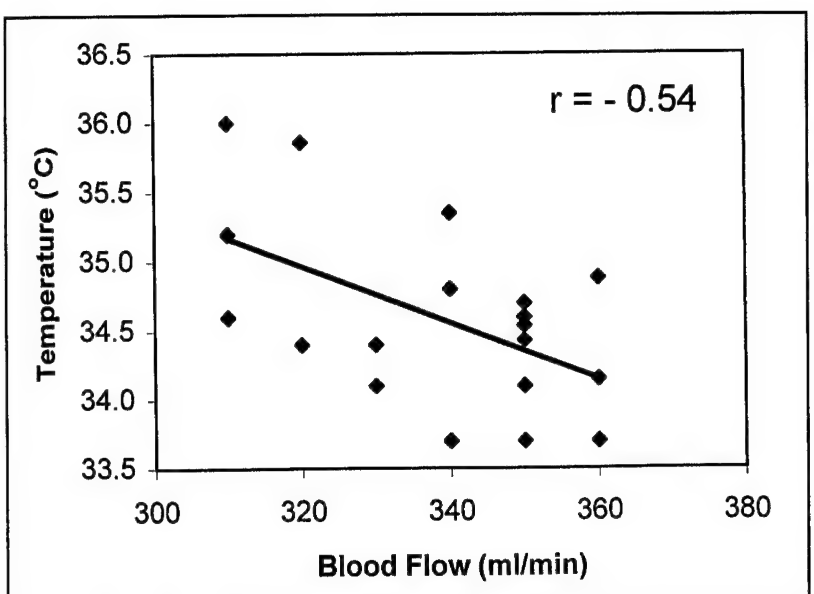


Figure 15. Graph depicting an inverse relationship between blood flow and graft heat, which is suggestive of that, inflammation and cell proliferation are generating heat.

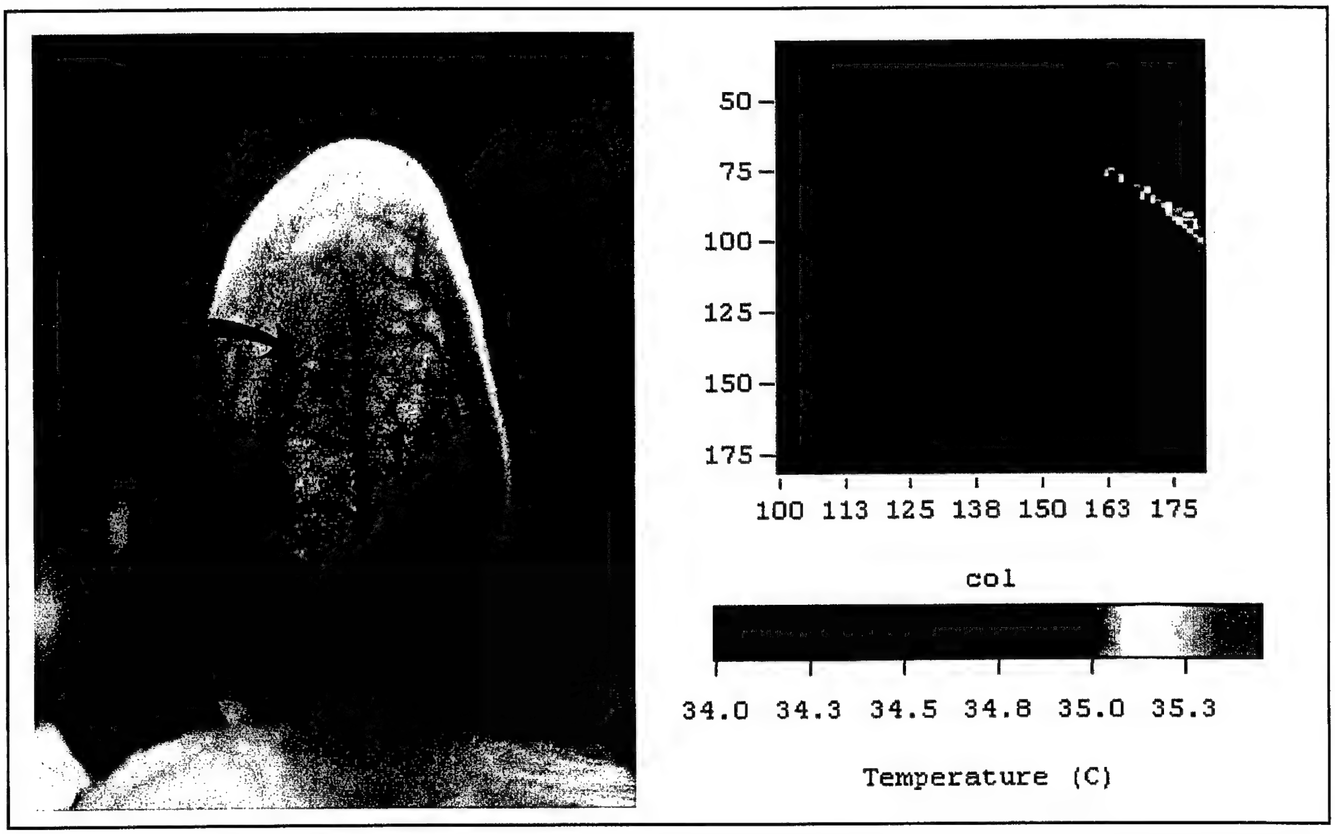


Figure 16. In Watanabe hereditable hypercholesterolemic rabbits on a high cholesterol diet, atherosclerosis is severe and extends to the arteries of the ear, where it is visible by routine phtography (left panel; ear lesions noted by curved arrows) , and by infra-red photography (right panel, a view of the region around the arrow in the left panel), which reveals thermal heterogeneity of the artery and the tissue.

ture. This will require prospective studies, and they will be challenging because of the lack of a universally accepted animal model of plaque rupture.

Eventually, thermal techniques could conceivably be combined with ultrasound catheters—or newer techniques, such as optical coherence tomography[22-24]—to provide both functional and anatomic information. Noninvasive studies, such as positron tomography and magnetic resonance imaging, could also conceivably be adapted to provide similar information, since inflammatory cells avidly consume glucose and lactate and it should be possible to image them using 18-fluorodeoxyglucose and 14C-lactate. Thermal magnetic resonance imaging has been used in some animal models to follow heat therapy for experimental tumors, and the current thermal resolution is in the range of 1° to 2°C, which may be sufficient for detection of the vulnerable plaque.[25]

Potential therapeutic implications:

If it can be shown that hot plaques do indeed go on to develop superficial thrombosis and/or rupture, they conceivably could be treated with angioplasty on the grounds that rupture and/or thrombosis is better performed in a safe, controlled environment.

Therapies other than angioplasty and stenting might include local treatment with anti-inflammatory drugs, antibiotics[26] (though we have found little association of plaque temperature with Chlamydia pneumoniae to date) or cytokines, such as TGF beta-1, which is anti-inflammatory while at the same time stimulate SMC proliferation and matrix secretion.[27] Yet another treatment might be gentle heating to induce apoptosis of

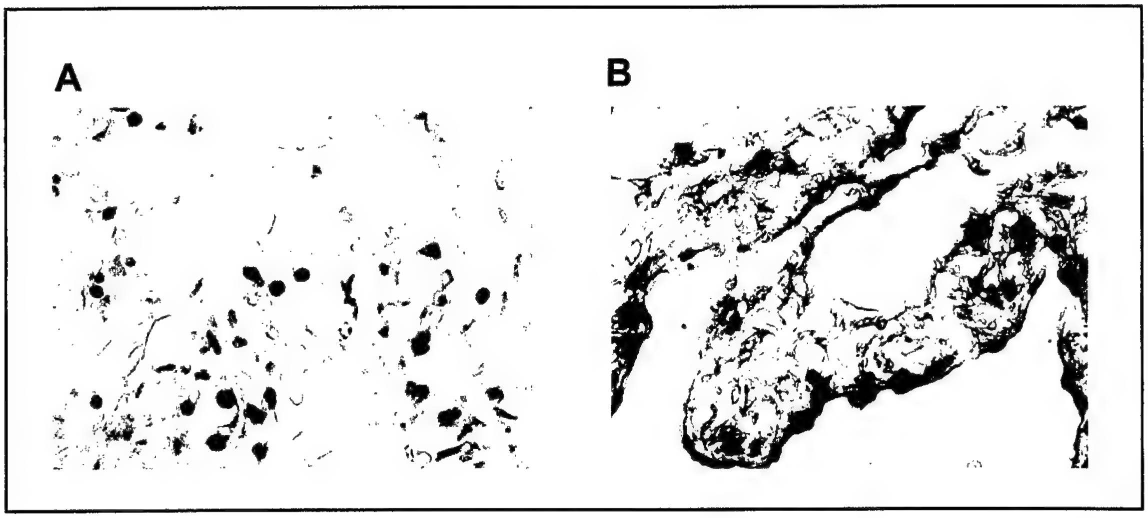
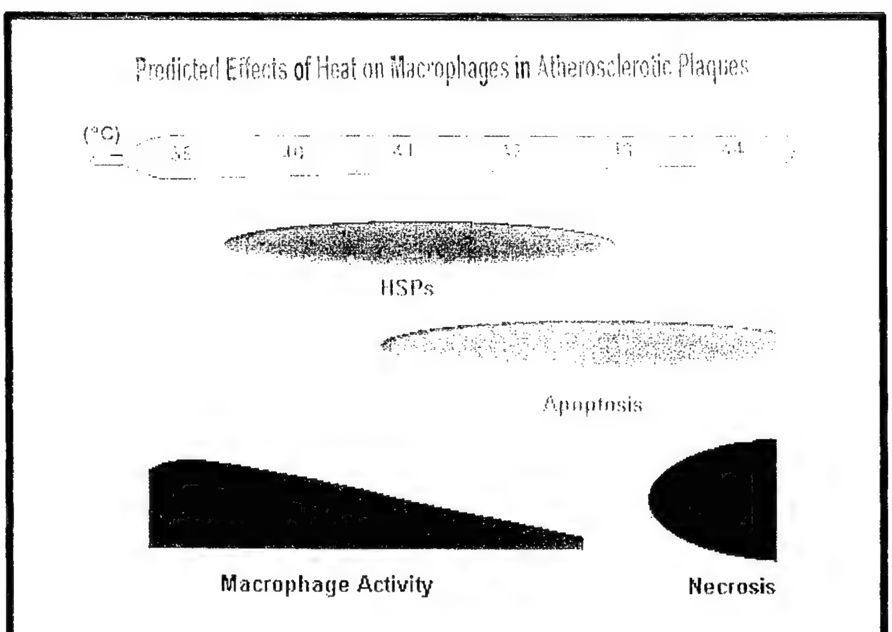


Figure 17. Thermal induction of apoptosis is illustrated in atherosclerotic plaque. Half was placed for 15 minutes in a 42°C incubator, while the other remained at 37°C. After 6 hrs. at 37°C, they were processed for histology. The cells undergoing apoptosis (shown by the dark nuclear staining) were identified using the terminal deoxynucleotidyl transferase reaction. The specimen maintained at physiologic temperature (A) indicates a significant incidence of apoptosis, but the percentage of cell undergoing apoptosis is higher in the specimen heated briefly at 42°C (B) particularly among cells with a macrophage morphology.

macrophages. Treatments such as these may temporarily stabilize the patient until such time as atherosclerotic regression in response to statins, aspirin, antioxidants, ACE inhibitors and lifestyle changes can take effect.

Interestingly, macrophage density has been found by Moreno et al to be the best cellular predictor of restenosis.[28] Therefore, heat-localizing techniques might also be useful to predict lesions at risk of restenosis and treat them. Heat localization may also aid in predicting, and then preventing, progression of inflammatory aneurysms.[29, 30]

Schematic 1.



Schematic Predicated effects on plaque macrophages of 15 minutes of heat. As shown in Figure 18, apoptosis actually begins at 41°C but peaks at 42-43°C. A 30 minute exposure is expected to double the percentage in apoptosis at 40-41°C.

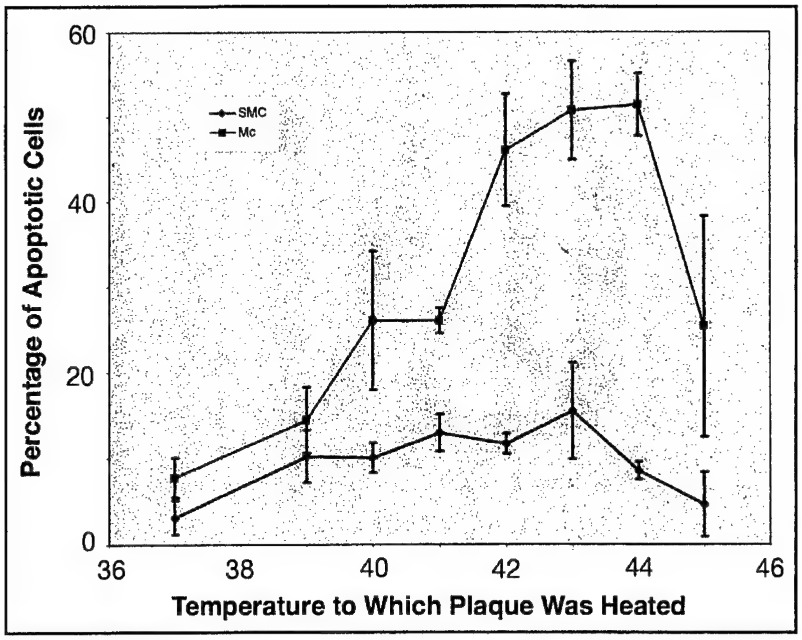


Figure 18. Thermal induction of apoptosis in macrophages. Living human carotid endarterectomy specimens, freshly harvested, were subjected to 15 minutes of incubation at the temperatures shown, followed by 6 hours at 37°C, then fixation and tissue processing for TdT reaction to detect overhanging ends of cleaved DNA, using the Trevigen kit, with confirmation in a subset of cells by electron microscopy and immunophenotyping. There is a slight increase in the frequency of apoptosis in smooth-muscle cells with heat, followed by the expected decline at necrosis-inducing temperatures (45° C. and above). In contrast, macrophages exhibit an abrupt increase in apoptosis beginning at 40° C. and maximal at 42° C. In another series, (not shown) apoptosis of macrophages began at 41°C and peaked at 44°C.

Clinical confirmation:

Very recently Stefanadis et al, have shown that patients with acute myocardial infarction studied with a thermistor on a catheter had substantial thermal heterogeneity (1.7°C) in their coronary arteries. Those with unstable angina varied by 0.8°C, stable angina 0-0.4°C, and normal arteries were uniform in temperature.[126-128] These studies, like our studies on the Watanabe rabbit aortas and central ear arteries, and the human AV grafts, establish that thermal heterogeneity is detectable *in vivo, in situ.*

Cellular thermogenesis

Food is converted to heat, work or growth (but mostly to heat, especially in the adult animal at rest). Gibbs' free energy comes from oxidation of carbohydrates, fats and proteins, coupled to reduction of NAD^+ to NADH. Next, NADH is oxidized by the mitochondrial electron transport chain, which is coupled to the generation of an electrochemical proton gradient across the inner mitochondrial membrane. This synthesis of ATP generates heat. More heat is produced when mitochondrial respiration is uncoupled from ATP synthesis, creating a futile cycle of proton pumping and proton leaking regulated by UCP1-

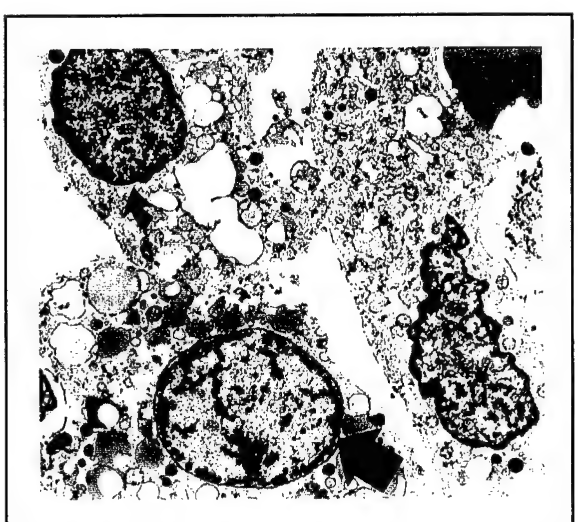


Figure 19. Ultra micrograph of three macrophages (arrows) in the human carotid endaretectomy specimen, denoting peripheral margination and fragmentation of nuclear chromatin, consistent with apoptosis.

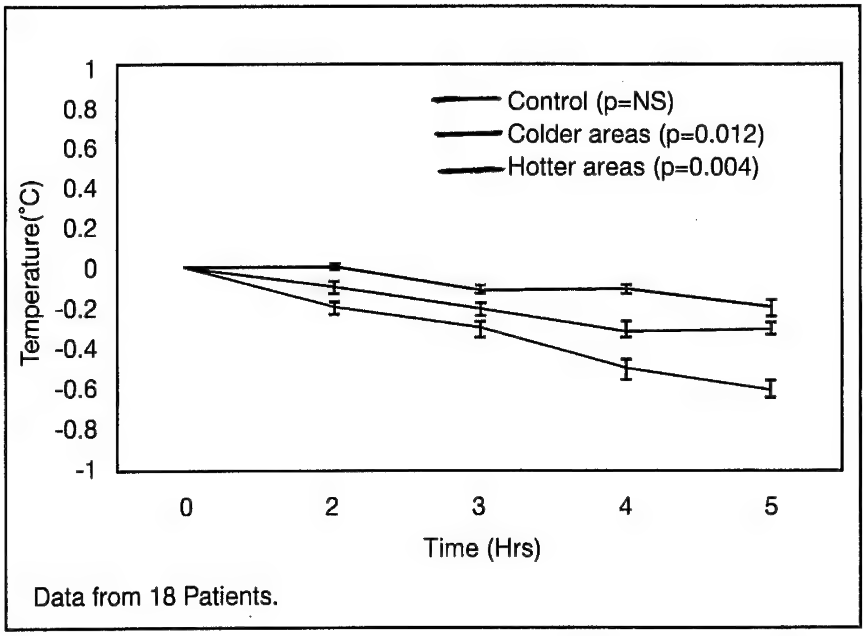


Figure 20. Gentle heating (15 min. 39-41°C) of living human carotid atherectomy specimens is followed by cooling, particularly in the warmest regions (≥37.50°C). Since these regions are mainly composed of macrophages one interpretation may be that heat down-regulates macrophage metabolism, a novel hypothesis but one that is suggested by the mRNA analyses summarized in table 1. The data above are pooled from 3 experiments (at 39, 40, and 41°C) done on 6 different plaques from 18 patients, because the 3 temperatures yielded very similar results.

UCP3. In addition, some O_2 consumption occurs outside the mitochondria, e.g. oxidative and degradative reactions in phagocytic cells.[31] Heat production is increased by activity, cold and feeding,[31-33] also by growth[34], and by activation (e.g., antigen binding, complement activation).[35]

Macrophage heat production

Few studies have measured the heat production of macrophages. Since macrophages appear to be the source of the heat in our atherosclerotic plaques, we were surprised that Thoren[36] found heat generation in neutrophils, lymphocytes and macrophages *in vitro* is modest[37,38] (compared to hepatocytes and adipocytes) perhaps reflecting the paucity of mitochondria, or lack of cell stimulation.

There is no published data on whether heat might be produced during the respiratory burst of stimulated macrophages but it is known that monocytes, macrophages and neutrophils, when activated, rapidly consume oxygen as a function of NADPH and NADPH oxidase[39], and that the subsequent generation of reactive oxygen species contributes to the destruction of microbes and other antigens. The generation of reactive nitrogen species also contributes. Heat production is usually proportional to glycolysis and lactate levels. We hypothesize that a great deal of plaque heat is generated during the respiratory burst. Since the burst is triggered by T cells, interferon gamma, TNFα, platelet-activating factor and endothelin-1, and inhibited by IL4, TGF β1, TGF β2 and IL10[40] these agonists should influence plaque heat. In a separate grant application we have proposed to elucidate the cells, cytokines and hormones that control heat generation in plaques.

Peroxisome Proliferator - Activated Receptors and macrophage functions

Peroxisome proliferator-activated receptor γ is a nuclear hormone receptor implicated in lipid metabolism, differentiation of monocytes, control of inflammatory gene expression and, recently, heat generation. PPAR γ is activated naturally by the prostaglandin 15d-PGJ2 and by the synthetic drug troglitazone, which is now given to approximately one million American diabetics to enhance their responsiveness to insulin.

PPARγ heterodimerizes with the retinoid X receptor and functions as a transcription factor, regulating such genes as adipocyte fatty acid binding protein, PEP carboxykinase, lipoprotein lipase and the thermogenic respiratory uncoupling protein UCP1.

PPARγ is expressed in many tissues but most highly in adipose tissue. Very recently, M Ricote et al[41]

reported that PPARγ is expressed at higher levels in activated macrophages than in monocytes, and that treating macrophages with 15d-PGJ2 decreases the production of iNOS and NO. C Jiang et al[42]) found that troglitazone inhibited production of inflammatory cytokines by macrophages activated by TPA and interferonγ. P Tontonoz et al and L. Nagy et al[43, 44] recently discovered that PPAR γ is induced by exposure to oxidized LDL, causing monocytes to differentiate into foamy macrophages with sustained expression.

Moreover, PPARγ promotes sustained expression of the scavenger receptor CD36. Thus, oxidization of LDL not only enables it to bind scavenger receptors but also converts a linoleic acid moiety into an activator of PPARγ, which in turn sustains scavenger- receptor expression, potentially contributing to a vicious cycle of foam-cell formation. Of particular interest to the present application is the report[45] that the PPARγ agonist troglitazone suppressed thermogenesis in culture adipocytes.

Also of interest is the fact that the related transcription factor PPARγ, which is activated by polyunsaturatred fatty acids, fibrates, and leukotriene B_4, stimulates beta-oxidative degradation of fatty acids, and inhibits oxidative activation of NFκB, and cytokine production in mouse spleen[46] PPAR-deficient mice show a prolonged response to inflammatory stimuli and PPAR activation causes apoptosis in macrophages[47]. Conversely, activators of PPARγ, such as the lipid-lowering agent fenofibrate, inhibit atherogenesis, in part by a lipid-independent action.[48] and decrease levels of iNOS and NO[49]. Finally, prostaglandins of the cyclopentone class, which activate PPAR, are reported to activate HSF1[50] and induce HSP70[51] and HSP32[52]

Mechanisms of fever production

As reviewed by Luhesi and Rothwell[53] local tissue injury causes the release of a number of cytokines, including IL1α, IL1β, IL6, IL8 and MIP (macrophage inflammatory protein-1). These stimulate the afferent nerve fibers to the brain, which synthesizes cytokines, prostaglandins and peptides that change the hypothalamic set point, change the metabolic rate and lead to thermogenesis (by shivering and nonshivering mechanisms) in addition to vasoconstriction and heat-seeking behavior. In the brain, IL1β and IL6 appear to act via PGF2α and corticotropin releasing factor (CRF). IL1α appears to act through synthesis of prostaglandin-E2, whereas IL8 acts through synthesis of CRF. Corticosteroids act (among other ways) by releasing lipocortin, which attenuates PG synthesis and probably synthesis of CRF as well. Derijk et al[54] found that depletion of macrophages by CL_2MDP markedly attenuated both production of IL1 and fever in rats, strongly suggesting a role for macrophages in the production of fever. Mice lacking the prostaglandin E receptor subtype EP3 are virtually incapable of fever.[55] The above mentioned studies measured core temperature; very little is known about local regulation of tissue temperature, but a recent paper[56] described induction by IL1B and TNFα of the energy-wasting, heat-generating UCP-2 in liver and muscle.

Utility of fever

The literature on the benefits and potential problems caused by fever is scanty and often poorly controlled. Mammals regulate temperature very tightly. Indeed, the morning fasting temperature of healthy persons varies by less than 1%, vs 50% or more for heart rate, blood pressure, cholesterol and glucose. Humans can detect a temperature change of 0.07°C.[31] Clinical data[57] generally suggest a beneficial effect of moderate fever, though high fevers can cause seizures and can increase cardiac work. Some of the confusion in the literature is clarified by recent studies: prior heat shock protects against subsequent heat, ischemia, or infection.

Heat shock proteins

The heat shock proteins – also called stress proteins – (Mr 105, 90, 70, 60, 32, and 27 KD) are expressed during development and differentiation, but the best known functions are the protection of cells against death from exposure to heat (generally 42 – 45°C), ischemia, hypoxia, toxins, growth factor deprivation, oxidation, inflammatory cytokines, chemotherapeutic agents, and high levels of NO[58, 59]. Hsp 27 protects

against apoptosis triggered by FAS and TNFα or ischemic injury in adult cardiac myocytes[60] as does hsp 70[61-63]. Heat shock also protects vascular SMC against NO toxicity[58, 64] HSP70 may act as a "heat sensor" by detecting heat-denatured proteins. In the rabbit reticulocyte lysate denatured proteins bind HSP70 releasing an eIF-2 kinase to decrease protein synthesis generally,[63] while HSPs are synthesized rapidly because the genes lack introns and the mRNA transcripts are stabilized by heat. The actions of the heat shock proteins themselves are not well understood, but in many organisms they facilitate folding of newly synthesized proteins, chaperone proteins into the mitochondria and prevent protein denaturation.

As reviewed by Polla et al,[65, 66] HSPs play a role in infection and inflammation. For example, HSP70 generally inhibits viral replication and the antiviral prostaglandins A and J activate heat shock transcription factor 1 (HSF1), an effect promoted by aspirin.[67, 68] Inflammation increases reactive oxygen species (ROS), which trigger (and are opposed by) 1) HSP32 (heme oxygenase, which inhibits iron-dependent oxidation, synthesizes CO, and increases cGMP), 2) HSP27 (which sustains glutathione levels) and, 3) HSP70 (which protects mitochondria and keeps them from triggering premature apoptosis). Inflammation also activates the transcription factor NF-κB, and this is blunted by activated HSF1[20] HSPs have also been implicated in the macrophage's processing and presentation of antigens to T cells and their promotion of the anti-inflammatory T cell subtype Th2, and protection of monocytes from oxidative stress.[69, 70]

Heat shock transcription factors (HSFs) are themselves constitutively present, and activated by oligomerization and hyperphosphorylation in response to stress. The transduction of the heat shock response involves c-Src tyrosine kinases, PI3 kinase, JNK, MAP kinases, (including ERK1 and JNK/SAP) and ribosomal S6 kinases[71].

Several authors have found that NO increases HSP-70 levels—for example, in rat heart[72] and in vascular smooth-muscle cells, via activation of heat shock factor1 .[58] In hepatocytes, NO increases HSP-70 mRNA and protein, as well as thermotolerance and resistance to TNF-alpha-induced apoptosis, in part by reduced generation of reactive oxygen species.[73]

Interestingly, when we performed a Gen Bank search of genes with 5' repeats of the heat shock element for HSP70B, we found perfect matches for iκB kinase, IL15, glutathione peroxidase, angiotensinogen, ubiquitin catalase, C-reactive protein and IL1β. Whether these genes are indeed activated (or repressed) by binding of HSFs to their HSE is not certain.

Many authorities now refer to heat stress proteins, believing that heat shock is rarely physiological. We hypothesize that heat shock indeed occurs in atherosclerotic plaques in vivo. After all, in a patient with a rectal temperature of 40°C and skin temperature of 30-36°C, there must be a focus of temperature greater than 40°C if 40°C is the net(core) temperature.

Does heat regulate immune functions?

We were surprised to find that placing a living human carotid plaque in a 39° or 40° incubator for 15 minutes (an exposure that causes little apoptosis) resulted in a decrease in plaque temperature after two subsequent hours at 37°C. The fact that the hottest spots cool the most, and that a similar (though later) fall in temperature occurred with indomethacin, suggests that the plaques have become cooler because of a decrease in inflammation, though indomethacin has numerous effects besides its anti-inflammatory and anti-pyretic effects.

Fever has received little consideration as an immune regulator. However we located a few relevant studies: Superoxide production by macrophages was increased at a higher temperature[74]. T-cell activation and production of leukocyte Migration Inhibitory Factor by mononuclear cells were enhanced *in vitro* by higher temperatures[75, 76]. Neutrophil function was also reported to be enhanced at 40° C [77]. Whole body hyper-

thermia (39-40°) enhanced immune responses in cancer patients[78]. In vitro, 41°C inhibited natural-killer T-cell activity though the response to IL2 and IF∝ was enhanced[79]

Conversely, high temperatures impaired mammalian natural killer-cell activity[80] and the generation of cytotoxic T-lymphocytes[81] Pre-heating rats to 41°C (105.8°F) resulted in HSP synthesis and a blunted release of IL1 in response to subsequent LPS (endotoxin), with improved survival. One hour of 41°- 43°C stimulated macrophages to produce prostaglandins, which act to decrease phagocytosis[18]. Exposure of rat peritoneal macrophages to 39° to 41° C. for 20 minutes decreased synthesis of TNFα and interleukin-1 [82,83]. Macrophage cytotoxicity was decreased by 60 minutes exposure to 40.5° C[84]. Heat shock had glucocorticoid-like effects on macrophages: blocking the induction by interferon gamma of the FC gamma R1 mRNA and transferring glucocorticoid receptors to the nucleus.[85] Heat exposure decreased release of TNFα by macrophages both in vivo and in vitro.[86] Taken together with our data from heating plaques and HL60 cells we infer the novel hypothesis that gentle heating is a broad, non-specific negative regulator of macrophage function, not dependent on apoptosis.

Nitric Oxide and temperature

Nitric Oxide, synthesized from arginine by NO synthase, plays important roles in neurotransmission, inhibition of thrombosis and leukocyte adherence, in vasodilation, and (in high concentration) in leukocyte cell-killing and microbicidal function. Recent work has also implicated NO in the regulation of temperature, mitochondrial respiration and apoptosis, as follows: Gourine et al[87] found that intravenous or intrathecal NO prevented fever. Mathai et al[87] found that NO donors lowered the core temperature of the awake rabbit; and inhibition of NO increased the temperature, mostly by vasoconstriction (decreased heat loss). NO may lower temperature by 1) vasodilatation (transferring core heat to the skin), 2) it's anti-inflammatory effects and 3) inhibiting mitochondrial cytochrome oxidase, thereby decreasing cellular respiration.[88, 89]

Heat shock decreases iNOS expression.

Heat shock inhibits cytokine-stimulated NO synthesis and expression of inducible NO synthase in hepatocytes, by preventing degradation of I-Kappa-B and NF-Kappa-B nuclear translocation.[90] In contrast LeGreves et al found that heat stress up-regulated neuronal NO synthase in the rat hippocampus[91], and Malyshev et al (1997) found that heat shock led to increased NO levels in rat heart.[92] Interestingly, Willoughby[93] and colleagues have found NO synthesis to be inhibited by CO, a product of HSP32 (heme oxygenase) and vice versa (refs).

Apoptosis

Apoptosis, or programmed cell death, is the process in which a single cell "commits suicide" in response to a severe stimulus such as infection, hypoxia, ischemia, oxidation, radiation, cytokines of the TNF∝ family, or heat stress. It is noteworthy that the same stimuli that induce apoptosis also induce heat shock proteins; presumably the heat shock protein response is too late or too little to prevent apoptosis, though this has not been proven.[62] Examples of apoptosis during embryogenesis include the elimination of surplus neurons and lymphocytes, and the remodeling of the hand bud into fingers.

Mediators of apoptosis in inflammatory cells are particularly relevant to the present application: FAS is a ubiquitous receptor which, when occupied by the FAS ligand, leads to rapid cell death. However, the related TNFα receptor and TNFα have a trophic effect on macrophages. Interestingly, in cells driven into apoptosis by FAS or TNFα, the mechanisms are different: TNFα impairs mitochondrial function, leading to an accumulation of reactive oxygen species, whereas FAS causes rapid protein phosphorylation (of substrates yet unidentified), resulting in activation of sphingomyelinase and ceramide-dependent activation of ras and, subsequently, of the endonuclease.

FAS and the TNF receptors have "death domains" that dimerize with proteins such as FADD and RIP.

These act through ras, JNK/SAP, ceramide and STAT1 in activation of ICE and caspase-3,[94-96] which cleave DNA into internucleosomal fragments. Other enzymes cleave lamins and focal adhesion kinases, and flip phosphatidylserine to the external side of the cytoplasmic membrane where it is recognized by annexin V. During this process the cell membrane remains intact, forming blebs as the cell shrinks, without releasing its proinflammatory and mitogenic contents. As a consequence, apoptosis is a "clean" death: the cell is engulfed by a neighboring cell ("amateur phagocytosis") or by a macrophage, with little or no subsequent inflammation.

Also relevant to the proposed experiments is the fact that NO is reported to inhibit FAS-induced apoptosis in immortalized human promonocytes, leukemia cells, and lymphoma cells, by a mechanism that does not depend on cyclic GMP, raising the question of whether redox effects or S-nitrosylation of proteins, or both, may be involved. Other factors which act to inhibit apoptosis include bcl 2 in multiple cell types and IL 15 in lymphocytes. IL 2 can induce or inhibit apoptosis in T-cells depending on the context [97].

Apoptosis and oxidation

Oxidation is a mediator of apoptosis even in response to non-oxidative stimuli.[98] Apoptosis induced by inhibitors of the plasma membrane NAD(P)H-oxidase involves Bcl-2 and calcineurin.[99] NAD(P)H oxidase is a redox sensor that can, depending on the ambient redox environment and the availability of growth factors, regulate plasma membrane calcium fluxes and signal for apoptosis through calcineurin.[100]

NO, a reactive species, appears to have dose-dependent effects on apoptosis, with lower doses inhibiting apoptosis in endothelial cells, for example, and high doses triggering apoptosis, in large part by the generation of peroxynitrite[101] Similarly, macrophages induced to synthesize large amounts of NO, by cytokine-induced iNOS, undergo apoptosis.[105]

Loven et al showed that after a 2 h exposure to 41.5° C, SOD activity of OvCa cells, but not of CHO cells, was increased. Thus SOD activity may be important in protecting cells exposed to heat and may play a role in the development of thermotolerance.[102]

Apoptosis in atherosclerosis

Bjorkerud[13] found about 15% of macrophages and T-cells in atherosclerotic lesions exhibited apoptosis compared to 1% of smooth muscle cells. Isner and colleagues[12] found an equal but low frequency (1-2%) of apoptosis in smooth muscle cells and macrophages. Bauriedel[103] found apoptosis in 28% of cells in unstable angina (mostly macrophages) vs 16% in patients with stable angina. Four weeks after stent implantation, macrophage proliferation and apoptosis were found in 33% of the cells.[104]

Thermal cell killing

The literature on therapeutic hyperthermia largely predates the literature on heat-shock proteins and apoptosis. Sensitivity to heat is highly variable, both *in vivo* and in cell lines. An increase of 1° C. is equivalent to a doubling of the exposure time. On average, half of the cells are killed by an hour of exposure to 44°C, but susceptibility is increased by cycling, acidosis, low ATP (but very low ATP results in necrosis, since apoptosis requires some ATP), nitroprusside[105], and age[106]. The mechanisms of thermal necrosis and thermal apoptosis are poorly understood, though protein denaturation and energy depletion have been postulated.

Thermal sensitivity of monocyte-macrophages

In theory, plaque macrophages and endothelial cells could be vulnerable to thermal apoptosis because they are low in PO_2 and pH and ATP, or they may be tolerant because they are "designed" to function in similar conditions in wounds and cancers. Elkon[107] found that granulocyte-monocyte stem cells did not survive as long as other cells did at 42.5°C. Cohen found no difference in thermal sensitivity between

airway macrophages and airway epithelial cells. Mangan[108] found that TNF α and IL1ß, which cause apoptosis in many other cell types, prevented macrophage (thermal) apoptosis. Prins described phagocytosis by macrophages following thermal apoptosis of fat, a finding reported in many other studies of heat treatment of tissue. The fact that macrophages induce apoptosis in normal cells in vivo[109] and the fact that macrophages engulf apoptotic neutrophils as inflammation resolves, suggest that macrophages may be resistant to apoptosis.

In short, there is no consensus as to whether macrophages are more or less sensitive to thermal apoptosis than other cells, and results applicable to the problem of plaque rupture are likely to be obtained only from studies of living plaque tissue.

CURRENT AND FUTURE RESEARCH

Overview:

Our project goals are to 1) understand what heat does to inflammation in the plaque and, 2) determine whether heat can be used to localize and treat vulnerable plaques. Because this field is new there are many more interesting questions than can be addressed in this grant. Yet because there is little guidance as to which questions are both important and answerable, the first year will be broad, with subsequent years focused on the mechanisms of selected projects. We will give priority to those projects that may aid in the development of heat-related diagnostic techniques or therapies. The outline below is expanded in our BAA application for FY '99 (under separate cover).

Project Goal 1: Determine whether heat regulates plaque inflammation and, if so, its mechanisms

Hypothesis 1a: *In plaque macrophages and T cells, heat in the fever range (39-41°C) down-regulates immune-related genes (eg IL1, IL2, IL6, IL8, MIP, TNFα) without causing apoptosis, and increases expression of anti-inflammatory IL10, IL13, PPARα and PPARγ.*

Hypothesis 1b: *Heat inhibits macrophage DNA synthesis, generation of NO, ingestion of foreign bodies, NADPH oxidase activity, activation of NFkB and secretion of matrix metalloproteases 1, 3 and 9, but increases MMP activity.*

Hypothesis 1c: *Heat-shock factors are activated and one or more heat-shock proteins are expressed in response to physiologic increases in plaque temperature (39 to 41° C.), especially in conjunction with acidosis, hypoxia, oxidative stress, or activation of PPARα alpha and PPARγ by eicosanoids or drugs (e.g., salicylate, fenofibrate or troglitazone).*

Hypothesis 1d: *Inhibition of macrophages by heat is mediated in part by activation of heat-shock factor 1 and induction of one or more heat-shock proteins (27, 32, 60, 70, 90 and/or 110); these responses protect macrophages against premature apoptosis.*

Hypothesis 1e: *More severe (e.g., 41° C.) abrupt or prolonged heat causes plaque macrophages to undergo apoptosis, while endothelial and smooth-muscle cells are more resistant.*

Hypothesis 1f: *The unusual sensitivity of plaque macrophages to thermal apoptosis at ≥ 41°C, is due to multiple mechanisms. Specific hypotheses suggested by the literature on hyperthermia therapy (mainly for cancer) are as follows:*

- *i) simply hotter than adjacent cells (when activated)*
- *ii) cycling*
- *iii) senescent*
- *iv) because of additional stresses such as oxidation (esp. via peroxynitrite), hypoxia, and acidosis*
- *v) because their HSP response is less than that of other plaque cells or is maximal at lower temperatures*
- *vi) because of heat-suppression of TNFα and NFkB, which (in macrophages) prevent apoptosis*
- *vii) because of MMP-mediated detachment of macrophages from their extracellular matrix*

(Judware R, BBRC ('98;2466:507) and MMP processing of TNFα and fas ligand (Guedez L, JC 1 1998;102:2002)

viii) because of inhibition by NO of macrophage respiration

ix) because of heat repression of glutathione peroxidase

x) because of activation of PPARα and PPARγ, or

xi) because of differences compared to other cell types in any of the other regulators of apoptosis such as bcl-2, bax, p53, ceramide, caspase 3, transglutaminase, etc.

Project Goal 2: Determine the natural history of the hot plaque.

A. Animal Models
1. Cholesterol-fed Watanabe heriditable hypercholesterolemic rabbits.
2. Cholesterol-fed, atherosclerosis-prone, Labrador dogs.

B. Human Studies
1. Natural History of the hot plaque in human arterio-venous dialysis grafts.
2. Aortofemoral Studies
3. Coronary Thermal imaging

Project Goal 3: Determine whether heat therapy can prevent plaque rupture and/or erosion.

A. Animals
1. Apo-E-deficient mice.
2. Cholesterol-fed Watanabe heritable hypercholesterolemic rabbits.
3. Cholesterol-fed atherosclerosis-prone Labrador dogs.

B. Human Studies

Clinical Trials of Heat Therapy:
1. Effect of local heating on arteriovenous hemodialysis grafts.
2. Superficial femoral artery (SFA) heating by ultrasound diathermy.
3. Coronary heating in patients with unstable angina who are high surgical risks.

Implications.

If it can be shown that hot plaques indeed are at high risk of rupture, it will be a strong impetus to attempt to develop a practical form of non-invasive thermal imaging. A catheter could also deliver heat to a circumscribed location, and cause selective macrophage apoptosis, it may "buy time" until other therapies can take effect. Another potential application is in blunting myocardial reperfusion—a well-established effect of experimental heat stress, and attributed to protection of cardiac myocytes by up-regulated HSPs. Another contributing factor would be a reduction leukocyte-mediated, oxidative and cytokine stress, due to an anti-inflammatory effect of prior heat exposure.

It is even conceivable that atherosclerotic inflammation could be differentially impacted by something as commonplace as the decision to use aspirin vs. acetaminophen (the latter having aspirin's antipyretic and analgesic effect without the anti-inflammatory effect). Finally, our studies may suggest that temperature could impact immune function in patients with cancer, organ transplants, autoimmune disease the maternal-fetal immune relationship, and wound healing.

Summary

In conclusion, we propose to extend our three novel findings: 1) atherosclerotic plaques exhibit marked thermal heterogeneity attributable to macrophage density and proximity, 2) macrophages are more sensitive than endothelial or smooth-muscle cells to thermal apoptosis, and 3) even lesser amounts of heat—below those required to trigger apoptosis—appear to down-regulate inflammation. We believe the effects of heat on the plaque should be identified and the mechanisms elucidated, and that this will lead to new diagnostic techniques and therapies.

PROJECT 2C

B. Thermal Imaging and Control of Inflammation

Project Goals:

1) ***Develop a catheter that localizes, quantifies and delivers heat.***
2) ***Develop non-invasive thermal imaging.***
3) ***Develop a non-invasive method of heating stents.***

Current and Future Research:

Overview: Our project aims are to **1)** develop the most accurate and precise catheter-based heat measurement and heat-delivery system, **2)** develop non-invasive methods of thermal imaging of atherosclerotic lesions **3)** develop a stent and non-invasive method of heating it.

Project Goal 1

A) Develop a catheter that localizes, quantifies and delivers heat

We propose to develop and compare several prototypes, each representing a different technology. Catheters utilizing infra red, thermocouples and thermistors will be built and tested. Alternatives will be catheters based on near IR, ultrasound, magnetic resonance, microwave, and liquid crystals, as described below.

REQUIREMENTS:

The minimal thermal resolution that will have clinical utility is predicted to be 0.2° C., and the spatial resolution should be at least 1 mm. The accuracy (validity in determining absolute temperature) is less critical than the relative temperature, but it should be at least 0.5° C. The minimum bend radius should be 3 cm. for peripheral work, and more flexibility will be required in the coronary arteries. The reproducibility of the measurements should be, from a physical standpoint, greater than 95 percent.

Moreover, the catheters should be 4 mm. or less in diameter for peripheral work and 2 mm. or less in diameter for eventual coronary work. The catheters should be relatively steerable and torquable and have a soft tip. They need to be durable and nontoxic and eventually producible for under $500 per catheter, if intended for single use, or sterilizable for re-use. The image acquisition should be acquired in two seconds or less, and the catheters and their equipment should be easy to use and relatively fool-proof from an operational standpoint.

1.A.1. Infrared (IR) Radiation: The electromagnetic spectrum includes radio waves, microwaves, infrared waves, visible light waves, ultraviolet waves, X-rays and Gamma rays (Fig. 21).

1.A.2. IR Thermography: Infrared thermography has long been used for non-destructive testing in a wide range of industrial disciplines. For example, IR thermography can be used to determine the condition of selected electrical components, motors, metal fatigue, etc.[143]

1.A.3. Medical IR Thermography: Thermography was investigated several decades ago for the detection of breast cancer. Thermographic liquid crystals and infrared-sensitive film were used to record asymmetries in the venous drainage from the breasts. The veins in most people are close enough to the surface to be identifiable by thermal mapping techniques. Markedly asymmetric patterns often indicated

revascularized promptly radiate warmth; whereas the ischemic areas remain cool. The grafts to these areas can then be adjusted to improve the flow. [114-117]

IR can provide an instantaneous and inexpensive estimate of metabolism and flow. Other techniques typically denote anatomy (e.g., CT or ultrasound), require angiographic contrast and radiation (e.g., angiography) or a radioactive tracer (e.g., nuclear imaging, as with a thallium or sesta MIBI scan) or require injection of a dye or other marker, together with blood sampling (e.g., green-dye dilution by the Fick principle). Similar information might be obtained by positron emission tomography, which can be used to determine flow and metabolism (with 18-fluorodeoxyglucose). However, this technique involves high-energy radiation, expensive equipment and a nearby cyclotron. Nevertheless, infrared had been little used clinically, and IR catheters have not been developed, presumably because of the large size, brittleness and expense of commercially available IR fibers.

1.A.4. Coherent Infrared Imaging Bundle for Cardiovascular Thermography

Rupture of atherosclerotic plaques – the main cause of heart attack and stroke – is not predictable. Hence even angiographic tests fail to detect many plaques at risk. Fatal plaques are found at autopsies to have been digested by many active inflammatory cells. Classically, swelling, red color, pain and heat characterize the inflammation. We have found that heat accurately locates the dangerous plaques. To develop a non-surgical method of locating these plaques, we propose to develop an infrared fiber optic imaging catheter.

1.A.5. The basics of IR fibers: Optical fibers have wide applications in telecommunication, medicine and other industries. Almost all of those applications use silica-based glass fibers. Optical fibers fabricated with silica-based glass have achieved the intrinsic attenuation limits of 0.2 dB/km at 1.5 mm. However the transmittance wavelength range of silica-based fiber is limited below 2.4mm which covers the visible and near infrared. For the biomedical applications, the mid-IR portion of the spectrum (3-12m) is of interest because that is the range of tissue heat.

IR fiber optics that transmit from 2m to 14m require entirely different materials for their fabrication. The materials include halide crystals and glasses, semiconductor crystals and glasses, heavy metal oxides and metals. Polycrystalline fiber optics are fabricated from the halides of silver, thallium, and potassium. Silver halides (AgCl-AgBr) are currently the most used. Chalcogenide glasses are those multi-component glasses containing the group VI elements S, Se. or Te as the network-forming anions and cations such as As, Ge, and Sb. The heavy metal fluoride glass optical fibers are similar in many ways to the silica-based fibers and only transmit IR radiation up to 4.5 mm. Single-crystals of Al_2O_3 or sapphire fiber optics can sustain high temperature and harsh environments.[145]

1.A.6. The coherent image fiber bundle: The coherent image fiber bundle is an assembly of fibers in which the coordinates of each fiber are the same at both ends of the bundle. It is also known as an aligned imaging bundle. Generally, a coherent bundle is used where spatial resolution is important. For example, it can be used to transfer the image plane remotely or locate an object. The imaging fiber bundles have been widely used in medical endoscopes.

1.A.7. The IR image fiber bundle:

Our first prototype fiber-optic imaging bundle consists of individual As_2S_3 chalcogenide glass fibers. This material transmits infrared radiation from 0.7 mm. to 7 mm. with relatively little energy loss. Only 100 m in diameter, the 1 meter long fiber bundle has a moderate flexibility. (Fig. 22) Combining it with a sensitive Indium Antimonide (InSb) infrared focal plane array (FPA) detector (Amber Radiance 1T, Infrared Imaging camera Raytheon, Goleta, CA), we can obtain the thermal graphic images *in situ.*

An *in vitro* experiment has shown that the fiber bundle is able to image the temperature heterogeneity of a homemade phantom. The device consists of four different temperature spots, each about 1.5mm in diam-

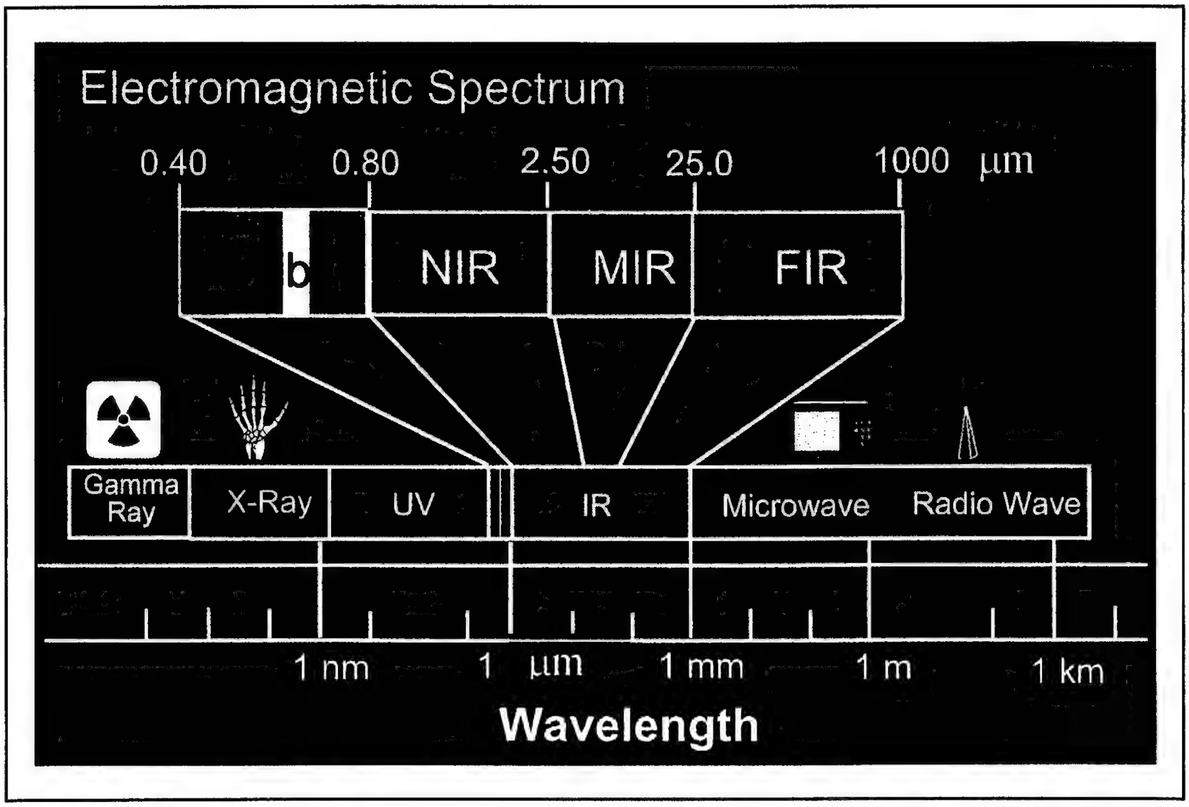


Fig.21 The electromagnetic spectrum
The IR region adjacent to the visible spectral region extends from approximately 800 nm to ca. 1000 mm or 12,400 – 10 cm^{-1} where the wavenumber (cm^{-1}) is often used in IR community. The IR region is further divided as[1]:

Near IR: NIR 12,500-4000 cm^{-1}, (0.8 – 2.5 micro meter)
Mid IR:MIR 4000 – 400, (2.5 – 25 micro meter)
Far IR: FIR <400 cm^{-1}, (>25 micro meter)

IR Thermography did not have the sensitivity of x-ray mammography for deep masses, many of whose temperature does not (directly) reach the skin and which do not create an obvious disturbance in the pattern of venous drainage. The technique thus fell into disfavor despite the enthusiasm of its early proponents. Recent developments in thermal imaging detectors, semiconductors, and high speed computers and imaging software have enhanced the accuracy and sensitivity of infrared thermography, making it more attractive as a non-invasive tool for medical and diagnosis. Medical IR thermography is a noninvasive diagnostic technique that allows the examiner to visualize and quantify changes in skin surface temperature, which maps the body temperature several mm deep to the skin, and is referred to as a thermogram.[144] Since there is a high degree of thermal symmetry in the normal body, subtle (0.02°C) temperature asymmetry's can be identified quickly and an explanation can be sought. Is there redness suggestion vascular abstraction or vasoconstriction? Is there warmth suggesting infection, cancer or inflammation? Is there phasic thermal "mottling" suggesting neural discharges (eg. sympathetic neuro-dystrophy)?

IR thermography can also be used to monitor the healing after burns, skin ulceration or grafting.
Infrared thermography has been used recently in determining the level of amputation in patients, with gangrene.[110-113] These surgeons found that a degree of coolness accurately demarcated nonviable tissue. Thermography has also been recently used to assess the completeness of revascularization during aortocoronary bypass surgery. An infrared camera is placed several feet over the open chest, and when the aorta is unclamped and normal blood flow is returned to the myocardium, the areas that have been

eter and 3-5 mm apart. The temperature heterogeneity of atherosclerotic plaques developed in the artery of the animal models will be studied with this prototype fiber optical imaging bundle.

An improved version of the IR fiber bundle is under construction. The new design will be smaller in diameter (3 mm OD), and a real system will couple the imaging bundle to our Raytheon-Amber Radiance-1t camera.

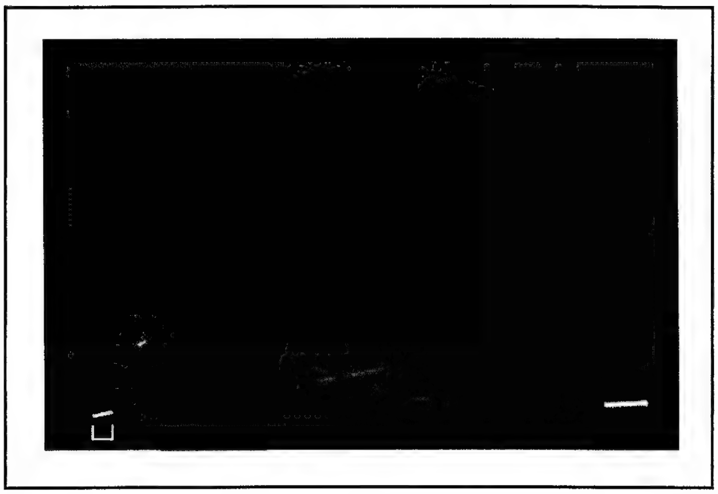


Fig. 22. The IR image fiber bundle consists of 900 individual infrared optics fibers. The total length of the fiber bundle is 100 cm.

The IR thermography catheter

1.A.8. Development of Catheters:

The ideal thermographic imaging catheter would provide high thermal and spatial resolution. The ideal image would be similar to that of an angioscope. The image would be acquired without computer reconstruction. The operation of the catheter would be safe, so that arterial flow would only have to be occluded for less than a second to obtain a snapshot. Real time heat imaging would also be important in studying the temporal stability of temperature heterogeneity (e.g., do hot spots come and go over seconds or minutes). Infrared imaging systems are attractive because of their nearly instantaneous speed of acquisition (a fraction of a second) and very high spatial (0.2 mm) and thermal (0.01° C) resolution, but the 100mm fibers currently available limit the spatial resolution of our IR bundle to 1/10 that of angioscopes and other endoscopes mode of 10mm glass fibers. We are working with Texas Instruments in an effort to obtain 25mm IR fibers. The goal is to develop a series of flexible, small (1 to 6 mm outer diameter), steerable, thermal imaging and heat-delivering catheters using a variety of infrared imaging fibers, encased in non-toxic polyurethane and/or polyethylene or other materials in current FDA-approved catheters. Several catheters have been designed as detailed in the Appendix. Most of them have a central lumen for perfusion, pressure measurements, and passage of wire guide. Most of the present designs will be equipped with a balloon to occlude blood flow and permit imaging of the vessel wall through an O_2/CO_2 mixture. One catheter uses an occluding balloon proximal to the imaging area. The other encases the imaging area in a long, soft balloon that is inflated in the artery and within which the infrared bundle is passed forward and back. Whereas the current prototype is forward-looking, one currently being built utilizes a conical, gold mirror at the tip, so that the catheter will give a circumferential (fish-eye) view of the artery. All the catheters can be used with a motorized pullback system, and with computer aided reconstruction.

Testing of the prototype

1.A.9. In-vitro testing: To test this catheter, we connected it to our infrared camera (Amber InSb FPA IR Camera NEDT=18 mk; 256 x 256 pixel; Raytheon, Goleta, CA 93117) and used it to image a homemade phantom. The phantom was constructed of 4 cm (long), 1.6 mm (diameter) columns of brass, wood, plastic, and lead, all painted black for uniform emissivity and reflectance. One end of this was heated on a hotplate. The differential conductivity of the materials provided different temperatures at the other ends of

these columns whose ends were imaged by the fiber bundle and camera, as shown in Fig. 23. Thermal resolution of about 0.2° C, and spatial resolution of about 0.2 mm were obtained. Note the grainy image caused by the fact that the prototype bundle consisted of only 900 fibers, a few of which were damaged. Images were taken with and without bending the catheter 180°, and while some attenuation was apparent it did not preclude imaging. Thus, our prototype-the first of its kind is quite encouraging.

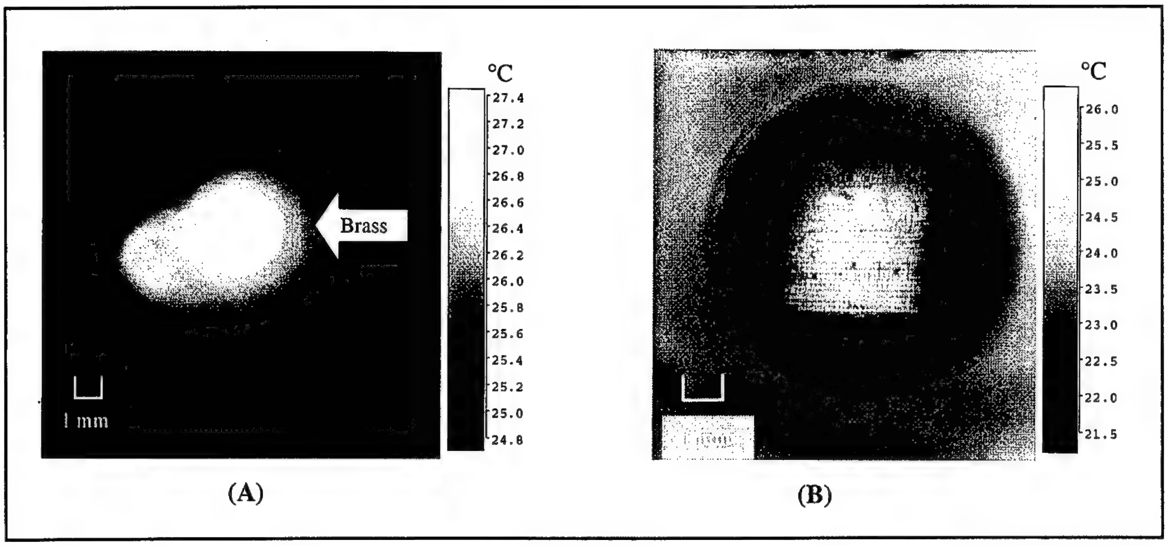


Figure 23. Thermal Imaging by IR Camera and by IR fiber Bundle
(A). The thermal image of a phantom taken with Amber Stirling cryocooled InSb FPA IR camera to show its thermal and spatial resolution. Each of the 4 circles representing thermal heat conduction from 36.2°C along 4 cm rods of brass, wood, lead and plastic in clockwise with brass the brightest. Thermal resolution of 0.2°C and spatial resolution of 0.2 mm are apparent. **(B).** Close up of the end of the brass column shown in panel A, but taken with the camera through a 900-fiber IR imaging bundle prototype shown in figure 21. Reasonably good spatial and thermal resolution are apparent.

1.A.10. Animal testing: We have just begun testing these catheters at the aorto-iliac junction of dogs made hypercholesterolemic by cholesterol feeding. These dogs were inbred by Dr. James T. Willerson and his assistant, Janice McNatt, and have a marked susceptibility to dietary cholesterol. They develop atherosclerotic lesions, not only at the aortic branch points, but also throughout the aorta and the coronary, femoral and carotid arteries, to the point of developing myocardial infarctions and strokes.
A 3 mm IR catheter is being constructed for similar studies in the rabbit aorta. In brief, they will focus on establishing the thermal and spatial resolution and safety of these catheters.

B) Thermocouples, thermistors, and alternative technologies

1.B.1 High Speed Multichannel Thermocouple / Thermistor Catheters:

There is no thermistor-tipped catheter commercially available which could be used to measure the temperature of the vessel wall. There are a number of commercially available catheters which monitor blood temperature in order to measure cardiac output (e.g. American Edwards/Baxter Swan Ganz catheter) by continuous thermo-dilution but this catheter can not measure temperature of specific plaques, lacks a guide wire and proximal occluding balloon and can neither image nor deliver heat. Nor do any U.S. Patents describe these features. Therefore we have developed two prototypes of multiple thermocouple and thermistor catheters. (Fig. 24-29). Obviously, the spatial and thermal resolution will not be quite as good or as fast with a thermocouple as with an infrared camera, and contact with the plaque surface will be re-

quired, but these catheters do not pose the problems of image degradation cost and limited flexibility that may impede IR fiberoptic imaging.

1.B.1.a.Thermocouple Balloon Catheter: (Fig 24-27) The prototype, we have built is a 4-channel thermocouple catheter (potentially expandable to 32 channels) designed to localize heat at 4 circumferential points on the vessel wall, in vivo. Obviously, the electrodes can be used to monitor temperature during heat therapy as well. Each bipolar electrode is connected to two wires (diameter 0.015 x 0.024 inch, Omega Engineering Inc., Stamford, CT) insulated and built in one sheet and silver soldered at the tip of the catheter; at the other side each sheet goes to one of the two channels of a dual thermocouple. We plan to replace it with a high-speed multi-channel thermocouple as the number of electrodes increases in future models. All the four electrodes were placed at equal distance from each other on the surface of an expandable balloon. The catheter can serve both in thermal diagnosis and therapy. There is a micro-aperture placed at the very end of the balloon to allow drainage of 5-7 ml saline/minute or 10-15 ml CO^2 /min. This is intended to assure a constant intra-balloon temperature during heat delivery, as it declines either by conduction through the wall or convection by blood flow. There is also a channel (or perfusion lumen) which enables the artery to continuously supply distal tissues while the catheter balloon is pressed against the artery to exclude blood flow and permit accurate thermometry.

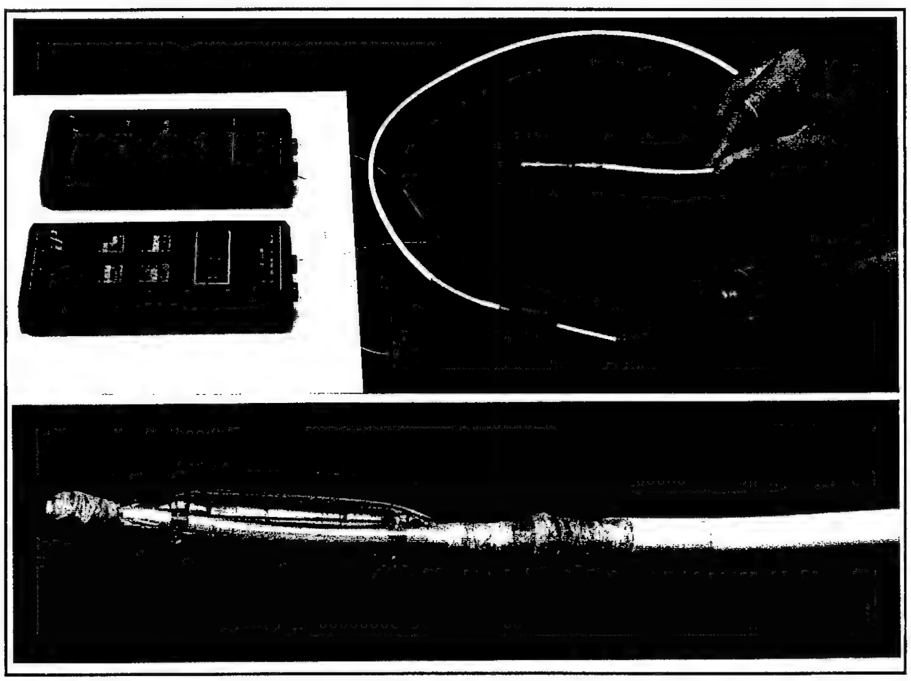


Figure 24. A) Photomicrograph showing the prototype thermocouple catheter. B) Photomicrograph showing the inflated tip of the catheter.

1.B.1.a.i. Heat Detection Mode: As illustrated in Fig. 25, the electrodes are placed in such a way that once the balloon is inflated each silver dot on the wires touches the arterial wall and measures temperature of one point on the endoluminal surface of the artery at a time. In order to use the same catheter for both diagnosis of thermal heterogeneity (localizing hot plaque), and thermal therapy, and also to avoid any bias in temperature reading of the plaque because of intra balloon temperature, it may be necessary to inflate the balloon with isothermal (37°C) CO_2 injection at a certain pressure and flow rate that assures the electrodes are contacting the plaque, and minimizing the heat exchange between the interior and the exterior of the balloon. Since the duration of thermal probing is estimated to take less than one minute, no significant increase in the blood PCO_2 is anticipated, and at any rate it is immediately buffered then expired through the lungs. Thermal resolution of the electrode is 0.2°C and its spatial resolution is about 0.5 mm with a response rate of <5 seconds.

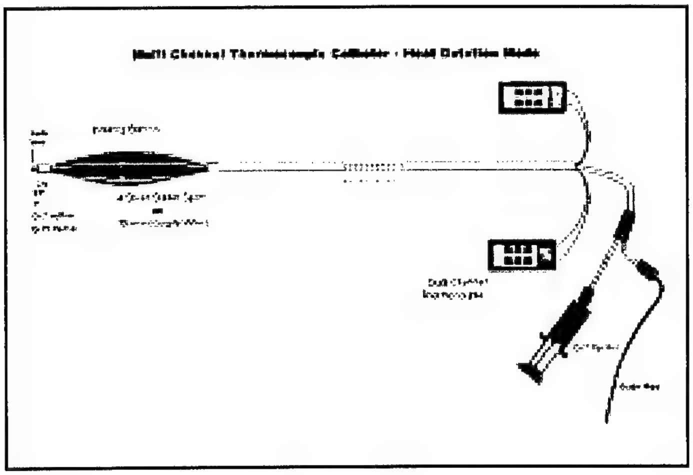


Figure 25.

1.B.1.a.ii. Heat Delivery Mode: (Fig. 27) Over the next year, we will explore the use of this catheter. After finding a hot plaque requiring heat therapy to induce macrophage apoptosis the system will simply be switched to the heat delivery mode with an appropriate heating protocol (39-42°C for 15 minutes). In this mode, after injecting hot saline into the balloon to inflate it, hot water will be continuously injected (5-7 ml/min) to keep the temperature constant. We have realized a flow rate up to 7 ml/min can retain the temperature at the desired level and counteract with heat washing out during the procedure. The heating temperature will be continuously monitored. Obviously 15-30 ml loading of normal saline won't be of any harm to the body. As mentioned earlier, a continuous perfusion model will be required to maintain blood supply to the distal part of the artery.

1.B.1.b. Thermistor Catheter: We have, most recently, built a dual-function thermistor device that monitors the temperature at specific points inside the vessel and can automatically heat the tissue to the programmed set-point (Fig. 28 and 29). Two resistance temperature devices (RTDs) were employed, one for measuring temperature and the other for heating. The temperature measuring accuracy is 0.25 °C, (range 0-50°C), with the response time about 0.5 second. The heating power is adjustable from 0 to 3 W. To measure and control the temperature the catheter has to closely contact the vessel wall, allowing no flow between them. For prolonged use, a perfusion model has been designed to meet this need. An insulated central tunnel is built in the expanded tip of the catheter, allowing the blood flow while the outer contact vessel wall as long as needed.

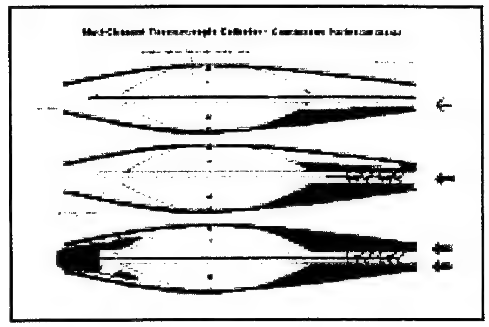


Figure 26.

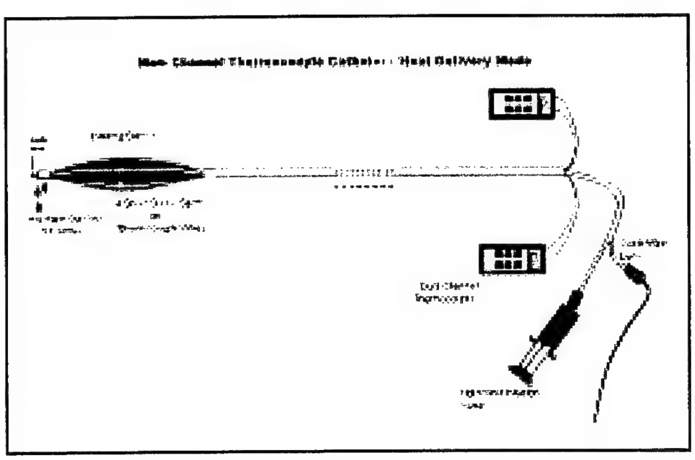


Figure 27.

1.B.1.c. Animal study: We will first test these catheters in rabbit aorta in vivo, mapping the thermal characteristics from the root to the abdominal aorta, and composing the results to those obtained by the IR catheter and needle thermometry. We will then study pig carotid and coronary arteries using the thermistor catheter. The risk that the 15 minutes infusion of saline will cause ischemia due to 5% hemodilution (anemia) is negligible.[118]

In fact, a recent paper calculated the threshold for anemia at 9mg/dl of hemoglobin: compared to a normal level of 15, the dilute blood carried less total O_2 but this was offset by the lower viscosity

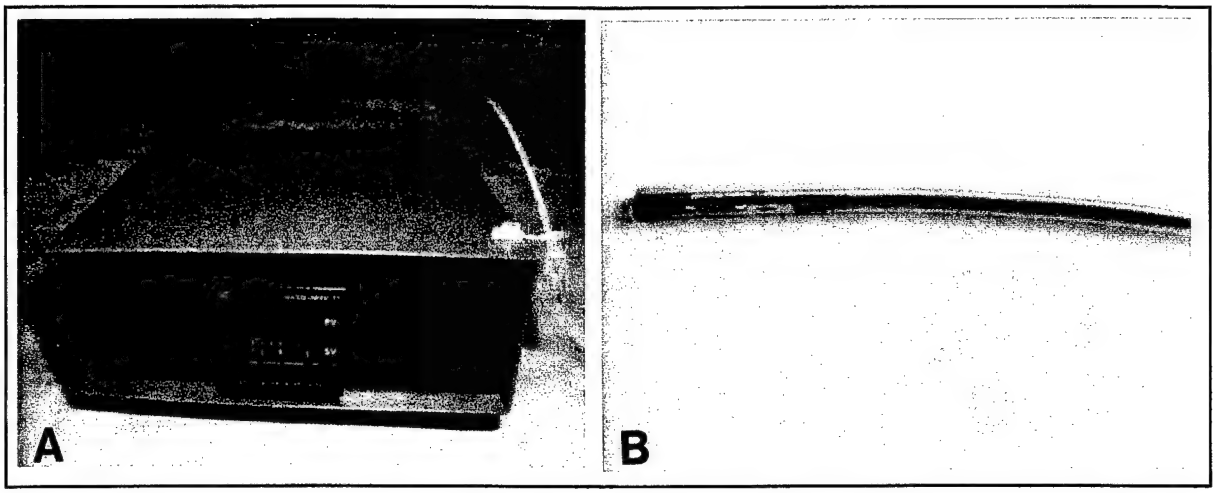


Figure 28. A) Photomicrograph showing the dual function electro-thermal device. It can measure and control the temperature through the same probe. B) Thermal catheter that contains two RTD's in the tip.

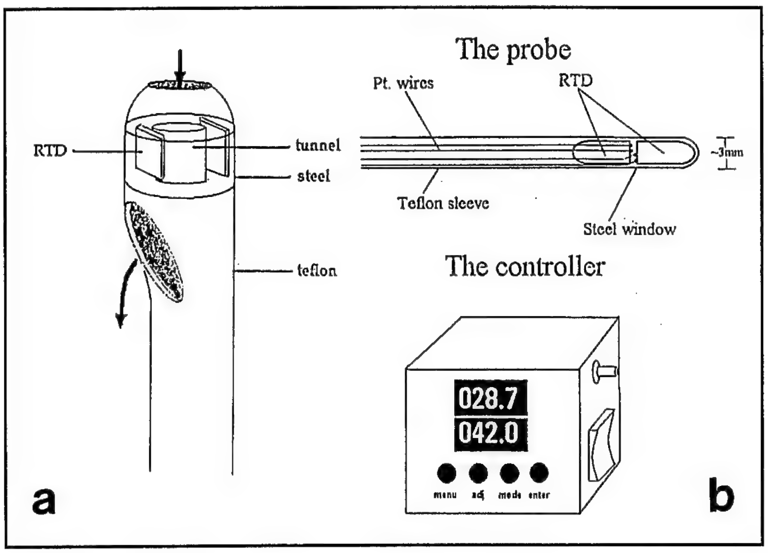


Figure 29. A) A prototype design for the thermister catheter with circulation tunnel for animal study. B) Dual-function electro-thermo-probe.

1.B.2. Balloon coated with liquid crystals or temperature-sensitive fluorphores

Thermographic cholesterol-based liquid crystals are available commercially. The problem with liquid crystal thermography is that spatial resolution changes rapidly as thermal energy spreads over the crystal. In preliminary experiments, we have coated a balloon with encapsulated liquid crystals and have demonstrated a visible color image with significant thermal and spatial resolution. Obviously, such a balloon would visual require monitoring from inside with an angioscope. A photograph of another experiment with liquid crystals is shown in Fig 30.

A variation on this theme would be a balloon internally coated with a temperature-sensitive fluorescent probe.. Temperature-sensitive fluorescent dyes are commercially available. The sensitivity of one of these has recently been described by Zohar and colleagues.[119] Using the temperature-dependent phosphorescence intensity of the rare earth chelate, Eu TTA (europhium III thenoyltrifluoro-acetonate), they achieved subcellular (e.g., 1 micron) resolution, with thermal resolution sufficient to see changes on stimulation of the cells' muscarinic receptors, a remarkable feat.[120] Clearly, painting an arterial wall or the inner aspect of a balloon inflated against the wall might prove to be a useful back-up approach if IR and thermocouple technologies prove unsuitable. No funds are requested for these studies.

1.B.3. Intra-vascular magnetic resonance imaging

Magnetic resonance imaging has recently attracted attention as a non-invasive technique for studying atherosclerosis. Preliminary work has also been described using magnetic resonance chemical shift imaging and spectroscopy of atherosclerotic plaque.[121-123] Because atherosclerotic vessels such as the aorta,

coronary arteries, and renal arteries are located deep in the body, surface coils do not produce optimal images. A small receiver coil near the vessel wall has superior signal/noise and spatial resolution. A few catheter MR receiver coil designs have been proposed for imaging the walls of large blood vessels such as the aorta.[124-126] Our collaborator, Alan Cohen, M.D., Professor and Director of Interventional Vascular Radiology at UT Medical School and Hermann Hospital, has published experiments using his intravascular MR catheter.[127] (Fig. 31). In separate experiments, our collaborators, Ponnada Narayana, Ph.D., Larry Kramer, M.D., and the PI and his team have used thermal imaging MR sequences to detect temperature differences of ~1.0° C in phantoms and tissue specimens. We hope to improve these techniques and apply them to Dr. Cohen's intravascular MR catheter. Our consultants Dr. Gerald Pohost and Dr Howard Kantor are pioneers in MRI and will help us in this respect. This project is in collaboration with Philips. No funds are requested for this.

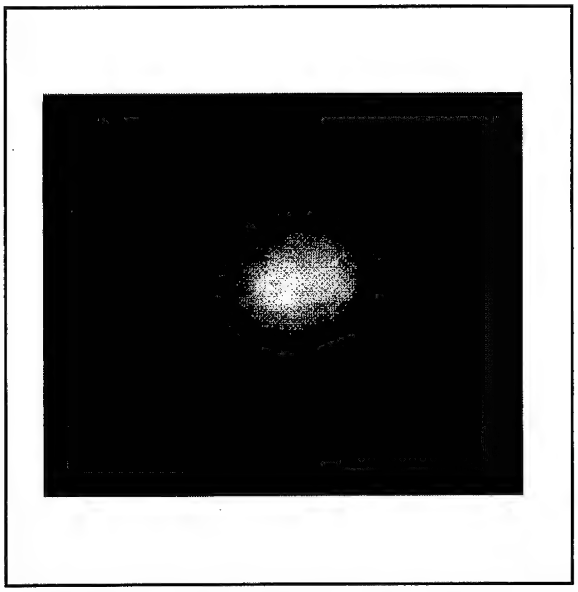


Figure 30. The color image of Liquid Crystal Sheet on the heated phantom described in Figure 22. The color part shown are the ends of brass (26.8°C) and lead (26.5°C) pole heated from the same base at 28.7°C.

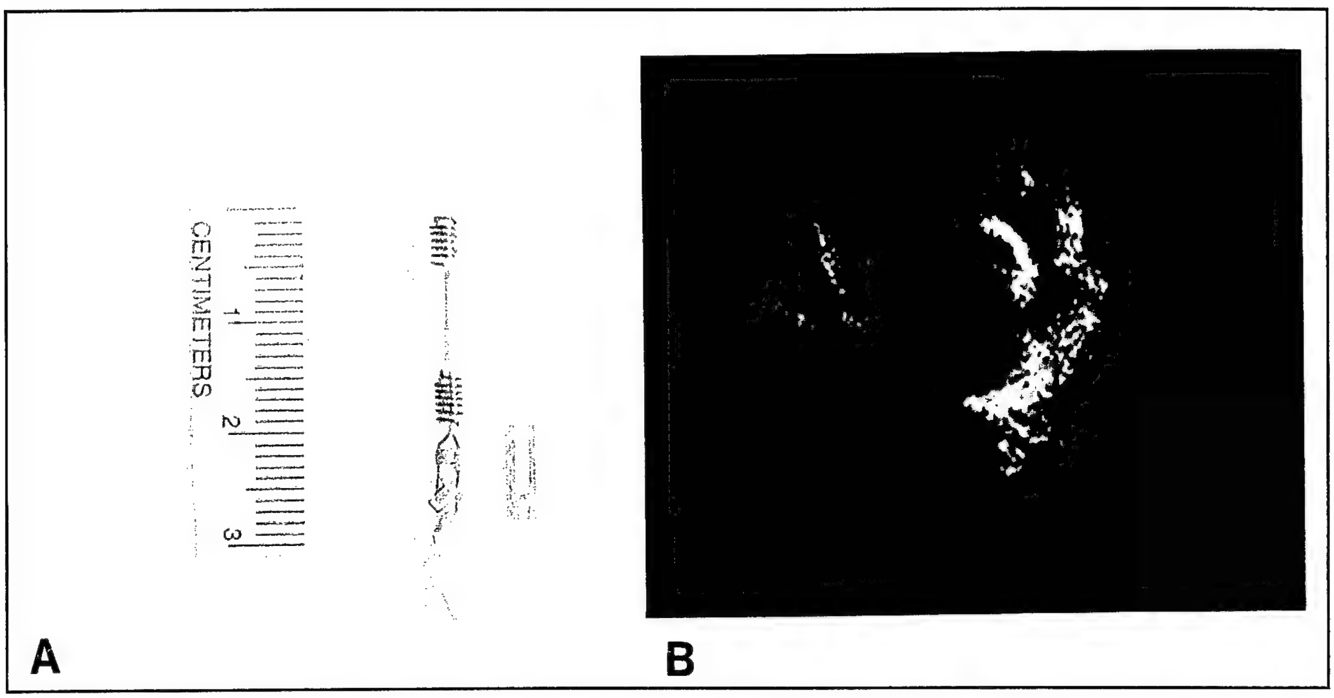


Figure 31. A) Photomicrograph showing the Intra vascular magnetic resonance receiver catheter imaging coil. B) In vivo image of live canine artery showing vessel wall architecture (2cm field of view).

Project Goal 2

Developing non-invasive thermal imaging

2.A. Thermal magnetic resonance imaging and spectroscopy

As mentioned above, magnetic resonance spectroscopy can identify some histochemical features of ath-

erosclerotic lesions.[121-123, 128-130] Several groups have adapted magnetic resonance to monitor the temperature of tumors after whole body hyperthermia.[131-135] Temperature alters T-1 and T-2 relaxation times of both water and lipids, and alters the resonant frequency of water attributable to variations in the molecular screening effects of electron clouds surrounding water protons. Thus, implanted thermocouples correlate with T1 relaxation time.[136]

The temperature-sensitive parameters that are measurable in MR experiments include the longitudinal and transverse relaxation times (T1 and T2), the molecular Diffusion Coefficient (D), and the proton chemical shift. In the case of relaxation times, the changes in T1 and T2 are tissue-specific and can often be difficult to measure reliably, i.e., different tissues have different relaxation parameters and their relative dependence on temperature changes is also different. On the other hand, for a specific tissue type such as the plaque, the changes in the relaxation times may be a useful parameter to measure. While the measurement of molecular diffusion coefficient (D) can be used as a thermometer (1), the diffusion weighted imaging techniques are extremely sensitive to bulk motion caused by physiologic activity, such as respiration and blood flow. Lastly, the rupture or bending of hydrogen bonds has been attributed as the mechanism for the change in chemical shift associated with alterations in local temperature. This chemical shift change is independent of tissue type and presents as unique advantage when compared to techniques that rely on changes in relaxation times. The measurement errors that can confound the chemical shift based results include primarily the following factors: (a) system drifts or instabilities, (b) magnetic field heterogeneity, (c) motion and/or blood flow within the vessels, and (d) temperature dependence differences between different tissues. We believe that while these are indeed formidable challenges, it is possible to develop methods that minimize the effect of these artifacts. We have appreciated similar efforts by others.[137-139] We intend to use spectroscopic shimming techniques to minimize field perturbations, use

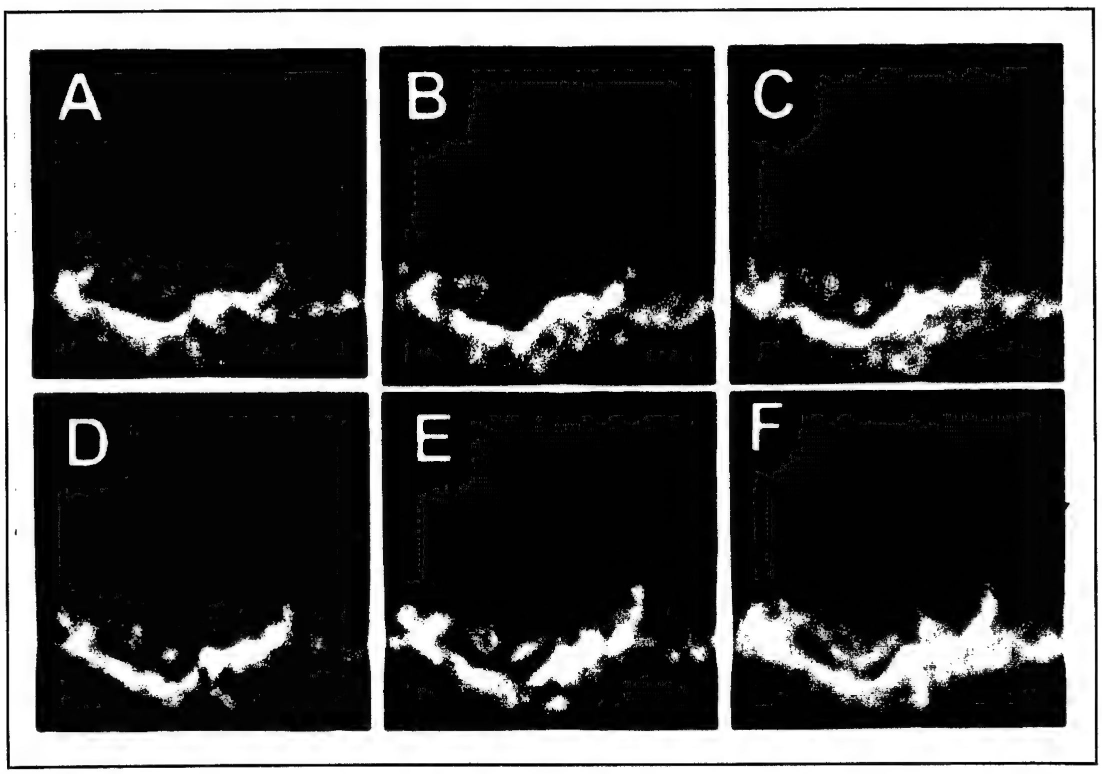


Figure 32. Magnetic resonance imaging of cholesterol-fed Watanabe hereditable hypercholesterolemic rabbit's aorta. After exposing, the aorta was placed in the test tube containing Dulbeco's Modified Eagle Medium (DMEM) and heated in awater bath of 42°C for 15 mins. MRI pictures, A to F were taken immediately, 3, 6, 10, 15, and 30 mins after heating respectively (e.g. during cooling to 37.4°C). the sensitivity of thermal sequences to temperature changes can be appreciated in the pictures A to C, whers the margins of the aortä are not as well demarcated (arrow) as in D to F (curved arrow).

navigator and ECG based gated techniques to minimize the effect of motion, develop techniques to reliably measure the system drift during the acquisition, and to develop specific methods to assess the temperature dependence differences between target tissues (e.g. vulnerable vs. stable atherosclerotic plaque).

2.B. In vitro study: Containers of water or lipid served as phantoms with varying temperatures. T-2 weighed images showed a loss of signal intensity with an increase in the temperature of the water. In the lipid phantom, signal intensity increased with temperature. A thermal resolution of approximately 0.8° C was obtained.

2.C. Animal study: Fig 32 shows our first attempt at thermal MR imaging of living aortic specimens of Watanabe hypercholesterolemic rabbit. We will be also testing the hypothesis in our own dog model of extensive atherosclerosis. Dr. James T. Willerson, co-investigator, and his associate Janice McNatt have developed a unique canine model of atherosclerosis. The lesions are histologically very similar to human plaques. Interestingly two of the dogs died of stroke. At autopsy there were extensive lipid-laden atheromas, mostly of the vulnerable types (type IV AHA classification) Therefore we plan to investigate the aortic and femoral plaques in these dogs using thermal MR imaging and spectroscopy to see whether plaques with higher thermal signals are indeed those with histopathological features of vulnerability. After that we will follow a cohort of animals, with and without extensive atherosclerosis, with periodic thermal MR to study the natural history of hot plaques.

Future plan

Future development will focus on new sequences. To this end, we have held a number of meetings with scientists at GE and Phillips, each of whom have indicated an interest in developing sequences for better thermal resolution. All of the scientists have agreed that this potential has not been fully explored. Recently, we have agreed with James Wang, Ph.D, Ruud de Boer, Ph.D and Raja Muthupillai, Ph.D, of Philips, to collaborate in this area as described in Dr. Muthupillai's letter. The research plan is detailed in our BAA application for FY '99 (under separate cover).

Project Goal 3

Developing a non-invasive method of heating a stent

3.A. Ultrasonically heated stent

3.A.1. Physics of the study: Our goal is to develop a means of heating selectively using an external source such as ultrasound radiofrequency, or an alternating magnetic field. A metal stent should absorb and/or reflect ultrasound waves to increase temperature of surrounding tissue, as the ultrasound wave reaches the interface of different media whose differences in cause part of the waves to reflect and scatter. In so doing a certain amount of the mechanical energy is transformed to heat.[140] In human studies, several reports have indicated that therapeutic ultrasound can heat bone more than surrounding soft tissue.[140-141] It is known that certain types of biopolymer absorb ultrasound waves and heat up more than other materials and we have found that ultrasound (as used in clinical diagnostic imaging) heats some plastic stents (A patent application filed on this topic is attached). Indeed, some plastics heated more than metal or tissue did.

3.A.2. Potential applications: We propose to use external heating of stents to prevent restenosis – a process of inflammation, proliferation of smooth muscle cells, accumulation and remodeling of extracellular matrix that causes narrowing of 20-30% of arteries after angioplasty and stenting. As explained earlier, we have shown that heating to 41°C can reduce inflammation in atherosclerotic plaque and induce apoptosis of macrophages. Dr. Robert Schwartz's laboratory at Mayo Clinic[146] reported that heat at 42° (for 15 min-

utes significantly induces smooth muscle cell apoptosis and reduces SMC proliferation. These two major components of restenosis, inflammation and proliferation,[142] may be treatable by simple, fast, painless and inexpensive ultrasound.

Future Plan: We intend to run a complete series of bench tests in order to address all the physical characteristics of the stent of choice as well as the preferred ultrasound radiation protocol. Also we plan to study the in vivo thermal behavior and eventually the therapeutic effect of non-invasively ultrasound radiation in preventing or reducing restenosis in comparison with control groups. Details are found in our BAA, FY '99 application.

Summary

In this report, we have described our progress in developing noninvasive and invasive means of identifying heat in tissues. Each technique has potential limitations and the optimal technique has yet to be defined. Practical non-invasive and invasive techniques would aid in the detection of vulnerable atherosclerotic plaques, and this knowledge may improve prognosis and, more importantly, allow for plaque-specific treatment to prevent heart attack, stroke and sudden cardiac death. Other possible cardiovascular applications include the identification of lesions at high risk of restenosis, or inflamed aneurysms at risk of rupture. Still another cardiovascular application might be the monitoring of infarct healing (an energy-requiring process) or myocardial reperfusion injury, a highly oxidative process that presumably emits heat.

Several of these invasive and noninvasive techniques have the potential to deliver precise amounts of heat to precise locations to cause necrosis or, even better, thermal apoptosis, and thereby prevent such unwanted consequences of inflammation as plaque rupture, thrombosis, aneurysm expansion, restenosis, and excess scarring.

Noncardiovascular applications can also be imagined, including diagnosis and treatment of vasculitis, rheumatoid and other autoimmune conditions, transplant rejection, infection and solid tumors. It should even be possible to identify areas of decreased heat, as might occur in the setting of necrosis, infarction or ischemia, or from a foreign body.

Local differences in tissue heat have received little attention, perhaps in part because accurate and inexpensive methods of detecting these differences have not been available. Such devices may now be more practical as a result of technologic advances and may become available as the awareness of the biological implications of local temperature differences in tissue are appreciated.

Project No. 2-D "Initial Evaluation of a New Axial Flow Pump, Inserted by Minimally Invasive Thoracotomy, to Maintain Cardiac Output in a Porcine Model of Cardiogenic and Hemorrhagic Shock"

Principal Investigator: O.H. Frazier, M.D.

Restatement of Project Objectives:

The purpose of this project is to investigate a novel approach to reducing mortality and morbidity due to injuries suffered by military personnel in combat zones. Specifically, the objective of this research is to evaluate the use of an implantable mechanical cardiac assist device, in conjunction with standard volume and/or blood replacement, for treatment of hemorrhagic shock resulting from injuries sustained in the combat setting.

The specific aims of this project are:

I. Refine the animal model of hemorrhagic shock in pigs.
2. Demonstrate decreased mortality in the animal model through application of circulatory support using a newly developed cardiac assist pump.
3. Demonstrate that this cardiac assist pump is lightweight, portable, easilv implanted and practical to use on the battlefield.

Progress Summary:

Texas Heart Institute's Associate Investigator, Dr. Branislav Radovancevic, Dr. Harald Eichstaedt, surgical research fellow. and Project Manager Mr. Dan Tamez traveled separately or to ether to San Antonio a total of three times in this reporting period to participate in animal studies of the parallel program at Brooke Army Medical Center (BAMC). Additionally, the THI group met with BAMC investigators to finalize the strategic plan and protocol for the studies at THI. *(Note.- As stated in previous reports, the vvork at BAMC is* not funded by the DREAMS program; BAMC investigators, *through separate funding, have pioneered the development of the hemorrhagic shock model in the pig. The approved DREAMS workscope includes travel to San Antonio to learn ftom the experience at BAMC, in order to minimize redundancy and reduce costs).* A total of nine experiments were conducted in San Antonio during this reporting period. Data form these studies were used as the basis for discussion and evaluation of the hemorrhagic shock model. and several key decisions were made regarding the surgery and instrumentation of this animal model.

Texas Heart Institute's Institutional Animal Use Committee approved the animal research protocol and the use of 15 animals. Ten pigs will be used as the test group and five as controls. Strategic decisions were made regarding the left ventricular assist system (LVAS). A commercially available centriftigal flow pump was selected for the test group.

A total of three studies were performed at the Texas Heart Institute. The primarv objective of these first studies has been to refine the hemorrhage model and to familiarize the researchers with the instrumentation and surgical procedure for this animal model. We presently feel that this animal model is reproducible and will continue the studies as projected.

Study Summaries:

DREAMS Study #1

This study was performed on 9-14-98 at THI. The animal was scrubbed and prepped in the standard fashion and transferred to the operating room. Prior to surgery each animal was sedated with Telazol (4.4 mg/kg IM), Ketarnine HCL (2.2 mg/kg IM) and Xylazine (2.2 mg/kg IM). Once the animal had been sedated an endotracheal tube was inserted, and an ear IV was catheterized. A patent IV was established and anesthesia/analgesia was maintained throughout the remainder of the surgery and studv duration with a Guaifenesin-Ketamine-Xylazine drip (2.2 mL/kg/hr). Following a standard surgical scrub of the abdominal and thoracic areas using chlorhexadine detergent the animal was transported to the operating room. Upon arrival to the operating room, the animal was placed in a dorsal recumbency position.

Under sterile conditions, a dual chambered polyvinylchloride catheter (Quinton) was placed in the right carotid artery. An oximetric Swan-Ganz thermodilution catheter was inserted into the external jugular and floated into the pulmonary artery for recording pressures, cardiac output, and continuous mixed venous saturation. The catheters were fixed to their respective vessels and tunneled to exit dorsally at the midline. A midline ventral abdominal incision was made and the spleen removed after double ligation of all vascular pedicles. A Transonic flow probe was placed on the superior mesenteric arterv and the abdomen closed in three suture lavers. The animals were connected to the appropriate transducers for a 30 minutes equilibration period. Once the transducers were calibrated the animal was hemorrhaged. The animal was hemorrhaged to a mean arterial pressure (AoP) of 40, This required the removal of 400 cc of blood and took approximately 70 minutes to accomplish. The FIO_2 was dropped to 30% and fluid administration was begun (2cc LRS/ 1cc blood loss). At approximately 30 minutes post hemorrhage the animal developed infrequent ectopic heartbeats and eventually fibrillated. The animal was defibrillated externally and was resuscitated according to ACLS guidelines. Thirty minutes after resuscitation started, the study was terminated due to intractable arrhvthmias. The animal was transferred to the pathology suite for a complete necropsy.

Study #2

This study was performed on 10-13-98. The animal was scrubbed and prepped in the standard fashion and transferred to the operating room. Prior to surgery each animal was sedated with Telazol (4.4 mg/kg IM), Ketamine HCL (2.2 mg/kg IM) and Xylazine (2.2 mg/kg IM). Once the animal had been sedated an endotracheal tube was inserted, and an ear IV was catheterized. A patent IV was established and anesthesia/analgesia was maintained throughout the remainder of the surgery and study duration with a Guaifenesin-Ketamine-Xylazine drip (2.2 mL/kg/hr). Following a standard surgical scrub of the abdominal and thoracic areas using chlorhexadine detergent the animal was transported to the operating room. Upon arrival to the operating room, the animal was placed in a dorsal recumbency position.

Under sterile conditions, a dual chambered polyvinylchloride catheter (Quinton) was placed in the right carotid artery. An oximetric Swan-Ganz thermodilution catheter was inserted into the external jugular and floated into the pulmonary artery for recording pressures, cardiac output. and continuous mixed venous saturation. The catheters were fixed to their respective vessels and tunneled to exit dorsally at the midline. A midline ventral abdominal incision was made and the spleen removed after double ligation of all vascular pedicles. A Transonic flow probe was placed on the superior mesenteric artery and the abdomen closed in three suture layers. The animals

were connected to the appropriate transducers for a 30 minutes equilibration period. Once the transducers were calibrated the animal was hemorrhaged. The animal was hemorrhaged to a mean arterial pressure (AoP) of 40. The equivalent preoperative and intraoperative procedures were performed as described earlier on the previous study. The animal was hemorrhaged to a mean arterial pressure of 40 in 35 minutes with a total blood loss of 1000cc. The FIO_2 was dropped to 30% and fluid administration was begun (2cc LRS/ 1 cc blood loss). At approximately 22:10 the animal developed bradycardia and eventually became asystolic. Resuscitation was attempted according to ACLS guidelines with no success. The study was terminated and the animal transferred to the chill room for a complete necropsy the following dav.

Study #3

This study was performed on 10-26-98. The animal was scrubbed and prepped in the standard fashion and transferred to the operating room. Prior to surgery each animal was sedated with Telazol (4.4 mg/kg IM), Ketamine HCL (2.2 mg/kg IM) and Xylazine (2.2 mg/kg IM). Once the animal had been sedated an endotracheal tube was inserted, and an ear IV was catheterized. A patent IV was established and anesthesia/analgesia was maintained throughout the remainder of the surgery and study duration with a Guaifenesin-Ketainine-Xylazine drip (2.2 mL/kg/hr). Following a standard surgical scrub of the abdominal and thoracic areas using chlorhexadine detergent the animal was transported to the operating room. Upon arrival to the operating room, the animal was placed in a dorsal recumbency position.

Under sterile conditions, a dual chambered polyvinylchloride catheter (Quinton) was placed in the right carotid artery. An oximetric Swan-Ganz thermodilution catheter was inserted into the external jugular and floated into the pulmonary artery for recording pressures, cardiac output and continuous mixed venous saturation. The catheters were fixed to their respective vessels and tunneled to exit dorsally at the midline. A midline ventral abdominal incision was made and the spleen removed after double ligation of all vascular pedicles. A Transonic flow probe was placed on the superior mesenteric artery and the abdomen closed in three suture layers. The animals were connected to the appropriate transducers for a 30 minute equilibration period. Once the transducers were calibrated the animal was hemorrhaged. The animal was hemorrhaged to a mean arterial pressure (AoP) of 40. Once again the equivalent preoperative and intraoperative procedures were performed as described in the first study. The animal was hemorrhaged to a mean arterial pressure of 40 in 30 minutes with a total blood loss of 1340cc. The FIO_2 was dropped to 30% and fluid administration was begun (2cc LRS/ lcc blood loss). Approximately 5 hours after the initial hemorrhaging was performed. 450cc of blood was returned to the animal to treat a hemoglobin that was less than 7 g/dl. At 2010 the animal developed some infrequent ectopic heartbeats and treated with boluses of Lidocaine. Once again at 02:00, 500cc of blood were returned to the animal to treat hemoglobin that wds less than 7 g/dl. At approximately 0600 the animal developed bradvcardia. The animal was resuscitated according to ACLS guidelines with no success. The study was terminated and the animal transferred to the chill room for a complete necropsy.

Planned Work during the Next Quarter:

Two additional studies have been scheduled in 1998. Our intentions are to use a commercially available centrifugal flow pump. We continue to collaborate closelv with Brooke Army Medical Center (BAMC) and the US Army Institute of Surgical Research and plan to attend at least one more of their studies.

History

Statistics compiled from battlefield casualties from the major wars of the 20th Century indicate that acute hemorrhage is the major cause of death in conventional warfare, accounting for about 50% of all fatalities. Evidence indicates that appropriate first aid and resuscitation in the far forward combat arena could avoid 20% of these deaths. It is also recognized that battlefield deaths increased as the length of evacuation time of the wounded soldier increased. In general, once the injured soldier reached a definitive treatment center, the percentage dying of wounds was low (2%). In civilian practice, hemorrhage from mechanical trauma, though less often fatal, remains a significant source of morbidity and mortality. Research focusing on agents that can minimize or prevent the deleterious effects of hemorrhagic shock and wound injuries may lead to improved therapy, thereby increasing the survival of injured soldiers and reducing the incidence of morbidity among the survivors.

Introduction

Prior to and during operations Desert Shield and Desert Storm, the Army developed and proved the concept of a forward area surgical team (FAST Team) having anesthesia and general surgical support near the front line. Thus, insertion of a simple cardiac assist device is now realistic. We will use a new device, which is small; easily portable, has a long battery life, and is resistant to adverse conditions such as dirt, sand, moisture, heat and cold. The device can be implanted with a minimum of technological support and is operable with minimal technical su arvision.

Project Objectives:

The purpose of this project is to investigate a novel approach to reducing mortality and morbidity due to injuries suffered by military personnel in combat zones. Specifically, the objective of this research is to evaluate the use of an implantable mechanical cardiac assist device, in conjunction with standard volume and/or blood replacement, for treatment of hemorrhagic shock resulting from injuries sustained in the combat setting.

The specific aims of this project are:

1 .Refine the animal model of hemorrhagic shock in pigs.
2. Demonstrate decreased mortality in the animal model through application of circulatory support using a newly developed cardiac assist pump.
3. Demonstrate that this cardiac assist pump is lightweight, portable, easily implanted and practical to use on the battlefield.

Progress Summary:

Texas Heart Institute's Investigators have traveled San Antonio to participate in animal studies of the parallel program at Brooke Army Medical Center (BAMC). A total of thirteen control experiments have been conducted, additionally one study was performed using a commercially available cardiac assist pump that has provided the investigators with encouraging results. Data from these studies is being used as the basis for discussion and evaluation of the hemorrhagic shock model.

Texas Heart Institute's Institutional Animal Use Committee has approved the animal research protocol and the use of 15 animals. Ten pigs will be used as the test group and five as controls. A

commercially available centrifugal flow pump will be used as the cardiac assist device for the initial test group.

Planned Work

Twelve additional studies have been scheduled in 1998. We continue to collaborate closely with Brooke Army Medical Center (BAMC) and the US Army Institute of Surgical Research to confirm the initial results from our earlier studies.

Project 2E: "Inhaled NO to Prevent Pulmonary Hypertension in Animal Models"

Principal Investigator: Robert Lodato, M.D.

Project was delayed by identification of personnel qualified to assist Principal Investigator and also by repeated submissions necessary to successfully achieve animal IRB approval. IRB approval has been approved and work has just begun on stated objectives. First and second quarterly reports for year #2 will provide initial scientific results.

<u>PROJECT II F</u> FOXHOLE STENTS TO PROTECT CELLS

INVESTIGATOR: Timothy Scott-Burden

<u>1st quarterly report</u>

Results: Vascular smooth muscle cells adhered more strongly to textured metal surfaces (sintered titanium microspheres) than endothelial cells. However both cell types appeared capable of attaching to the metal surface when initially seeded on to it. Adhesion was enhanced by the culture of cells in the presence of ascorbic acid. The latter promoted the elaboration of extracellular matrix (ECM) material by both cell types with a marked increase in the collagen content of the ECM (2-5% in the absence of ascorbic acid to 20-35% in the presence of the vitamin). The main difference between the two cell types was observed when monolayers were subjected to flow generated shear stress. A centrifugation assay was developed to mimic flow and the effect of shear stress. This assay system demonstrated a greater loss (5% of total cells seeded) of endothelial cells from the textured metal surface occurred as compared to smooth muscle cells (2%) under identical conditions up to a maximum shear stress of 75 dynes/cm. Cell loss occurred during the initial exposure to shear stress and after 45 min of continuous shear no further cells (endothelial or smooth muscle) were lost from the metal surface. Cells that had been exposed to 45 min of shear and then returned to static conditions were still resistant to re-exposure to shear stress after 24 hr. Overall, the growth and adherence of cells to the metal surface was superior to previous studies using materials such as textured polyurethane.

<u>2nd quarterly report</u>

Results: Two isolates of smooth muscle cells were tested for their ability to grow and adhere to the surfaces of metal stents. Scanning electron microscopy (SEM) was used to assess the adhesion of the two cell populations to the stent surfaces following exposure to shear stress (25 dyne/cm) for 1 hour at room temperature. The cells in panels A,C and E synthesize twice the amount of elastin/elastic fiber as the cells shown in panels B,D and F. SEM images were taken at magnifications as indicated and there is a clear difference in the ability of the two smooth muscle isolates to adhere to the stent surface.

<u>3rd quarterly report</u>

Results: Previously (see report for period ending 4/30/98) we had shown that smooth muscle cells adhered to polished stent materials. Also enhanced elastin synthesis enabled them to better resist sloughoff due to shear stress. In the studies reported here both smooth muscle and endothelial cells were tested for their ability to adhere to biomaterial surfaces that differed in the extent of their processing (roughness). Cells were seeded onto the biomaterials at the same cell density and attachment was monitored by SEM 48 hrs after seeding. Both cell types adhered well to the unprocessed bimaterial and also to the polished material. The poor adhesion to partially textured (satin finish) materials was unexpected and currently we have no explanation for this observation. The SEM images were typical of those obtained with three samples of each biomaterial seeded with the two cell type.

<u>4th quarterly report</u>

Results: Endothelial cells were transduced with a commercial plasmid pCI neo containing full length coding sequence for bovine ligamentum nuchae tropoelastin. After transfection, transduced cells were selected by culture in medium containing neomycin (G418) and then tested for their adhesiveness compared to control non-transduced cells. The centrifuge adhesion assay has been developed in the Vascular Cell Biology Laboratory and allow us to process up to 16 samples at time. When endothelial cells transduced with tropoelastin were compared with control cells in this assay system there was less than a 2% loss of transduced cells from the glass substrata used as compared to a 25% loss for control cells. Western blotting of cell lysates from control and transfected cells suggested that a truncated form of elastin was being expressed by latter.

Project: IIIA "Role of Sentrin in Apoptosis"

Principal Investigator: Edward T.H. Yeh, M.D.

Apoptosis, programmed cell death, plays an important role in reperfusion injury and in cell death caused by radiation, trauma and hypoxia. We have discovered a novel ubiquitin-like protein called sentrin- I which interacts with the cell death domains of Fas and tumor necrosis factor receptor- 1. Over-expression of sentrin- I in cells can inhibit anti-Fas and tumor necrosis factor induced cell death.

We found that sentrin- I can be covalently attached to other proteins and majority of sentrin-modified proteins are localized in the nucleus. Sentrinization, i.e. sentrin modification, is a novel pathway for protein modification distinct from ubiquitination, which has been. shown to play important roles in cell cycle progression, inflammation, and cell death induction. We have identified the activating enzyme complex (Aosl[Uba2) and the conjugating enzyme (Ubc9) for the sentrinization pathway. In this budget period, we demonstrated that PML, a tumor suppressor protein involved in the pathogenesis of acute promyelocytic leukemia, is covalently modified by all sentrin family members. Acute promyelocytic leukemia arises following a reciprocal chromosome translocation t(15;17), which generates PML-retinoic acid receptor a fusion proteins (PML-RARct). We have shown previously that wild type PML, but not PMLRARA, is covalently modified by the sentrin family of ubiquitin-like proteins. In order to understand the mechanisms underlying the differential sentrinization of PML vs. PML-RAR(x, extensive mutational analysis was carried out to determine which Lys residues are sentrinized. We showed !hat Lys65 in the RING finger domain, Lys I6O in the B I Box, and Lys490 in the nuclear localization signal contributes three major sentrinization sites. The PML mutant with Lys to Arg substitutions in all three sites is expressed normally, but cannot be sentrinized. Furthermore, the triple substitution mutant is localized predominantly to the nucleoplasm, in contrast to wild type PML, which is localized to the nuclear bodies. Thus, sentrinization of PML, in the context of the RING finger and the BI box, regulates nuclear body formation. Furthermore, we showed that sentrinization of PML-RARA could be restored by over-expression of sentrin, but not by retinoic acid treatment. These studies provide novel insight into the sentrinization pathway.

We are now testing the function of sentrinization using PML as a model substrate. We wi 'II determine whether sentrinization is required for PML's tumor suppressive activity and its ability to enhance the transcription co-activator activity of CBP. Insights derived from these studies should significantly improve our understanding of the protective role of sentrin in apoptosis signaling and in other biological processes.

Appendix:

Kamitani, T., K. Kito, H.P. Nguyen, H. Wada, T. Fukuda-kamitani, and E.T.H. Yeh. 1998. Identification of three major sentrinization sites in PML. J. Biol. Chem. 273:26675-26682.

III B. Mechanisms of Cell Adhesion and Reperfusion Injury.

Investigator Name: Edward T.H. Yeh, M.D.

Cell adhesion plays a critical role in the attachment of leukocytes in all types of reperfusion injury. Major mediators of this are E selectin and L selectin. We have developed several inhibitors of the selecting. One small molecule inhibitor has been successfully used in ameliorating myocardial damage in canine models of ischemia reperfusion injury.

The extend the relevance of this inhibitor to the battle field and trauma setting, we have begun to test whether these inhibitors can inhibit recruitment of inflammatory cells in a mice model. We have developed an in-vivo model of inflammatory cell homing and have used the model to demonstrate selective usage of adhesion molecules in the recruitment of inflammatory cells. In this budget period, we further refined this in vivo macrophage homing model using a murine monocyte/macrophage cell line, RAW, as a surrogate. We showed that the homing of RAW cells to the atherosclerotic plaque is quite similar to those of the peritoneal macrophages. Furthermore, the window for detecting a reduction in cellular adhesion is readily achieved using RAW cells as a surrogate. Adhesion of RAW cells to the inflamed artery can be prevented by pre-treatment with anti-a4 antibody. We are in the process of testing a small molecule inhibitor of a4 to determine whether homing of RAW cells to the inflamed artery can be inhibited. Validation of the biological activity of small molecule selectin inhibitors would have significant implication for better treatment of battle field injury.

Project Number/Title: 3C "Novel Methods of Protein and Gene Loading in Macrophages"

Principal Investigator Name: James T. Willerson, M.D.

We have developed a novel monocyte-derived macrophage homing model for the study of macrophage homing to atherosclerotic plaques in Apolipoprotein-E deficient mice that develop spontaneous atherosclerosis by 30-40 weeks of age. We have used macrophages loaded with fluorescent microspheres injected intravenously into 40-week old Apolipoprotein-E deficient mice to study their homing to atherosclerotic plaques. Macrophage attachment to vascular tissue is believed to be centrally important in the development of atherosclerosis, its initiation and its progression, as well as in the fissuring or ulceration of atherosclerotic plaques that lead to abrupt thrombosis and myocardial infarction and strokes. Thus, a better understanding of the mechanisms responsible for macrophage homing, of their physiological functions within atherosclerotic plaques, and the elucidation of methods that prevent macrophage accumulation in vascular tissue should prove extremely useful in human medicine.

In our studies in the past year, we have demonstrated that monoclonal antibodies to the a-subunit of the a4pl integrin and against intracellular cell adhesion molecule (ICAM-1) reduce macrophage homing by 75% and 85%, respectively, as compared with isotype-match controls ($p < 0.05$). Furthermore, combined monoclonal antibodies directed against these two integrins reduce macrophage homing by 85-90% ($p<0.01$). More recently, we have shown that Troglitazone, a medication used to treat Type 11 diabetes and a gamma PPAR modular also reduces macrophage homing to atherosclerotic plaques in this model by approximately 40% ($p < 0.04$) when given repetitively to mice by intragastric lavage. It is our plan in the coming year to determine whether combinations of these interventions might markedly reduce or prevent atherogenesis in this murine model. The demonstration of a markedly protective effect by these interventions should have very important implications as regards methods to reduce the progression of atherosclerosis in humans.

Project Number: 3D "Genes Regulating Wound Healing and Susceptibility to Oxidative Injury"
Principal Investigator: Eric Boerwinkle, M.D.

The principal investigator and administrative support staff at UTHSC have made several attempts to submit the information required for IRB review and approval. The format of the current protocols are such that the Army IRB committee cannot approve them. The project has been on hold up to the point of the annual report as we continue to try and meet the Army's requirements.

PROJECT III E: "Over-Expression Of Vegf In Smooth Muscle Cells To Enhance Endothelial Regeneration"

INVESTIGATOR: Timothy Scott-Burden, Ph.D.

<u>1st quarterly report</u>

Results: Transduction of endothelial cells with VEGF cDNA resulted in their expression of several new and some, not unexpected properties. Transduced endothelial cells (expression of VEGF was confirmed by western blot analysis of conditioned media) exhibited enhanced rates of growth as compared to mock transfected cells. This result might be expected since VEGF is a potent, specific growth factor for endothelial cells. However, surprisingly the expression of VEGF by the transfected cells also enhanced their sensitivity to basic fibroblast growth factor. The levels of expression for FGF receptor type 1 (FGFR1) were however reduced in cells that had been transfected by VEGF cDNA. Therefore the increased sensitivity to FGF2 observed in VEGF transduced endothelial cells was not due to a simple upregulation in specific FGF receptor number in the transfectants.

<u>2nd quarterly report</u>

Result: Plasmid constructs containing VEGF-GFP coding sequence were introduced into smooth muscle cells using a commercial star burst dendrimer preparation SuperFect. Expression of the fusion protein was confirmed by western analysis of condition medium from transfected cells. Transfected smooth muscle cells were established in coculture with endothelial cells (which express receptors for VEGF) and they accumulated green fluorescent in their nuclei. Lysis and western analysis of endothelial cells grown in coculture with pREP9 VEGF-GFP transfected smooth muscle cells confirmed that the former had taken up the fusion protein construct, presumably via receptor mediated endocytosis. These data suggest that the fusion protein is functional at least in respect to receptor recognition. Expression of GFP alone does not lead to secretion of product or uptake into a nuclear location by endothelial cells.

<u>3rd quarterly report</u>

Results: Bovine smooth muscle cells were transduced with pREP9 FGF2 plasmid constructs using similar procedures to those already described for pREP9 VEGF constructs. DNA was introduced into cells using either Helios gene-gun delivery or by means of commercial preparations of star burst dendrimer molecules. Cells were grown under selection conditions (250μg/ml Neomycin) and surviving colonies expanded and tested for expression by western analysis. FGF2 transduced cells exhibited enhanced growth rates in cultured as compared to cells transduced with pREP9 vector alone (mock-transduced cells). In addition FGF2 transfectants were growth responsive to added exogenous VEGF in a manner analogous to that observed when cells were transduced by VEGF genes and then exposed to exogenously added FGF2.

Treatment of mock-VEGF- and -FGF2 transduced cells with IL-1β resulted in a concentration dependent production of NO. At low levels of cytokine, FGF2 transduced cells produced close to maximal levels of NO (50 nmole/10^6 cells) whereas mock-transduced cells produced only +/- 5% of this amount. Similar observations were evident for cells transduced with VEGF

<u>4th quarterly report</u>

Results: VEGF-GFP was purified from the culture insect cell conditioned media using HPLC chromatography and fluorescence detection. Proliferation experiments with endothelial cells using this material (5n/ml) were carried out in a comparative fashion using commercial hrVEGF. Although growth stimulation was submaximal with the commercial material in served to demonstrate that the VEGF-GFP did not stimulate the same cells under similar conditions. However conditioned media from smooth muscle cells transduced with VEGF-GFP cDNA stimulated growth of endothelial to a similar or greater extent as seen with commercial hrVEGF.

Collection of conditioned media from mammalian cells transfected with VEGF-GFP cDNA and grown in a hollow fiber cartridge (Cellco), was initiated in order to prepare large enough quantities of the fusion protein. Hollow fiber cartridges are incorporated into pump-driven flow loops and in this way secreted products (in this case VEGF-GFP fusion protein) accumulate in the medium resevoir. Cell growth and nutrient status of the cells is monitored by measuring the lactic acid content of the circulating medium. Medium was harvested at four daily intervals and pooled for processing over a heparin affinity column.

Project III-F: "Nontoxic Peptide Enhancement of DNA Uptake"
Principal Investigator: David A. Engler, Ph.D.

Year one progress has proceeded to advance all three technical objectives of this project to various extents. First, we have shown that basic fibroblast growth factor (bFGF; FGF-2) is capable of enhancing delivery of plasmid DNA into cells grown in culture as evidenced by the bFGF-mediated enhanced expression of activity from exogenously supplied reporter genes. This directly impacts on technical objective (1) of this project and has been exemplified by data as reported in quarters 1 and 2. Although we have been able to consistently show that FGF enhances the delivery of plasmid DNA to cells under the conditions being studied, we have also observed inconsistencies in the quantitative nature of such delivery schemes. We believe that a major reason for this inconsistency is the formation of an insoluble precipitate between the FGF-2, polylysine and DNA used to form the DNA delivery vehicle. We have accordingly devoted effort to try and decipher the underlying causes of these non-productive molecular interactions in order to minimize this confounding factor on data interpretation. This effort has taken the form of trying to reproducibly form molecular conjugates of FGF-2, polylysine, and plasmid DNA under conditions that do not promote visible molecular conjugate aggregation, and/or the removal of such precipitate if formed. Under such conditions we inevitably observe a corresponding decrease in reporter gene activity from the transfected cells. We have further attempted to design better quantitative assays for measuring the stoichiometry between molecules making up "active" preparations of molecular conjugates (quarter 2), as well as genetically engineered the FGF-2 molecule to minimize certain types of chemical reactions that may potentially act to promote such heterogeneity in sample preparations (quarter 2). Additionally, we have recently constructed new molecular tools to help us in our effort to biochemically purify or enrich our FGF-PL-DNA samples in the biologically "more active" form (quarter 4). All of these efforts directly address technical objective (2) of this project.

Initial studies have been designed and tested for feasibility with regard to furthering technical objective (3), the elucidation of FGF-mediated transport mechanisms that allow this enhanced delivery of DNA molecules into cells, presumably through FGF-dependent entry ways such as the FGF receptor (FGFR) system. These ongoing studies were first reported in quarter 3 and revolve around the use of genetically engineered fluorescent derivatives of the FGF-2 molecule. We are taking advantage of the naturally fluorescent properties of a protein known as green fluorescent protein (GFP) by genetically coding for a fusion protein between FGF-2 and GFP. This fusion molecule is then either delivered to the cultured cells exogenously, or the gene for this fusion protein is transfected into the cells to promote its endogenous production and transport out of, and then into, the cultured cells by either an autocrine or paracrine mechanism. We then attempt to visually observe the molecular trafficking of these molecules from the cell surface to the cell nucleus in an attempt to better understand the mechanism of FGF/FGFR-mediated gene delivery. This aspect of project III-F will be a major focus of year two goals and ties in well with project III-H (Fluorescent molecular imaging) and may give important insights into FGF-DNA transport that can also be utilized to further the goals of project III-G (Role of FGF-2 in mutagenesis and carcinogenesis).

Project III-G: "Role of FGF2 in Mitogenesis and Carcinogenesis"

Principal Investigator: David A. Engler, Ph.D.

Year one progress has been directed at establishing experimental paradigms under which to study what effects addition of exogenous, physiologically relevant doses of FGF-2 has on the uptake of DNA fragments (similar in size and structure to those that might be liberated under conditions of cell wounding and nuclear disintegration) into viable cells. The technical objectives were to determine if such DNA uptake could actually happen under conditions that mimicked the pathophysiological state encountered at sites of wounding, and whether this uptake results in an enhanced somatic cell mutation rate in surviving cells. We have demonstrated that FGF-2 is capable of binding to DNA fragments (quarter 1) and that the addition of both FGF-2 and DNA fragments into the culture medium of growing cells that express the FGF receptor increase the abundance of DNA in the nucleus of viable cells (quarter 4). These data were obtained in support of technical objectives (1) and (2) of this project. They do however not go far enough as to unequivocally answer the question of whether this FGF-mediated increased abundance of exogenously added DNA fragments in the cell nucleus have actually integrated into the host's nuclear (chromosomal) DNA. This would be an absolute requirement for the possibility of a mutagenic event to exist. It is also a fact that our experimental paradigms, although a good model for what may actually occur at wound sites, have not directly shown that FGF and DNA released from the wounded cells are capable of directing nuclear transport of these DNA fragments into healthy neighboring cells. These are aspects of the project that will continue to be addressed in year two, as well as experiments designed to address, in a quantitative fashion, whether or not this DNA fragment uptake results in pro-mutagenic events in surviving cells at sites of wounds (technical objective 3).

Substantial reagent preparation has occurred during year one in terms of cell lines engineered to express various levels of the molecules thought to be necessary for FGF entry into cells (e.g. reported in quarter 2), as well as the testing of several fluorescent imaging models to test the various hypotheses this project is designed to evaluate (reported in quarters 3 and 4). A major advance to this project should occur in year two and in conjunction with project III-H when we actually will be able to acquire enhancements to our current microscopic image analysis capabilities. We plan to continue to advance the technical objectives of this project by focusing on the BrdU-labeled DNA fragment model as reported in quarter 4 with respect to the labeled DNA's potential to recombine with host chromosomal (non-labeled) DNA, and if measurable, to expand that population of cells that shows enhanced recombination to assess the extent of somatic cell mutagenesis.

Project III-H: "Luminescent and Flourescent Molecular Imaging"
Principal Investigator: David A. Engler, Ph.D.

Year one progress on this project to evaluate the potentials of diagnostic imaging via luminescent and fluorescent molecules has advanced two of the three technical objectives specified. Early work focused on the ability of luminescent reporter systems to differentiate cell phenotype, both in terms of cell lineage and physiological state. These results were reported in quarters 1 and 2 and centered around the use of antibodies and lectins to specific cell surface markers that allowed the types of determinations just mentioned. Thus this data directly impacted technical objective (1). However, the type of reporter systems used in these experiments utilized a diffusible chemiluminescent substrate that presumably was able to diffuse away from the site of antibody interaction with cell surface molecule before photon liberation and light emission, and thus were not ideally suited for high resolution imaging applications, even though they possessed the desirable quality of high sensitivity to subtle physiological changes occurring within a cell and registered by the differential display of specific cell surface molecules. Later progress, as reported in quarters 3 and 4, directly impacted technical objective (2) in that it allowed direct testing of several of the same antibodies that were tested under luminescent conditions under analogous conditions using fluorescence as the detection mode. The fluorescent methodology has enabled the high resolution imaging of cellular structures under the microscopic conditions employed. While this is in itself not surprising given the fact that this type of immunohistochemical fluorescent detection methodology is well tested, we were able to show that this type of imaging capability could be carried out on living cells (quarter 4). These data allowed for the comparative evaluation of both luminescent and fluorescent reporter molecules for molecular imaging purposes, a key goal under technical objective (2). Initial studies would therefore suggest that the fluorescent methodology lends itself well to ease of use and potentially could be quicker in cell labeling studies given the fact that no additional substrate or cofactors are needed for reporter molecule activity, but that it may suffer in sensitivity issues - depending on the mode of image (photon excitation and emission) capture. This factor of course illustrates an extremely important point. That is, the utility of any future luminescent and/or fluorescent molecular imaging is directly proportional to the sensitivity and ease of use of the device(s) used to capture the image. Since most of our studies have been at the cellular and ex-vivo tissue levels, microscopic and/or gross image capture (for example on X-ray film) have been employed. The identification of weaknesses in our current microscopic imaging technology has hindered the high resolution, real-time imaging that we are striving to obtain. With regard to this aspect, we have been evaluating numerous commercial instruments (microscopes, CCD's, digital cameras, optics, etc...) that we feel will be necessary for us to obtain to enhance our ability to use these types of molecular imaging techniques to their fullest, as well as act as an enabling technology to more accurately predict the type of clinically useful image capture devices that will be necessary to translate this basic research tool into a useful diagnostic tool suitable for bedside evaluation. The image capture device that ultimately will be used will of course also dictate to a large extent which of these molecular imaging techniques would be most useful under any given situation. We plan on continuing this line of research into year two and expand the studies originally defined for this project to include intra-cellular molecular trafficking of fluorescent molecules. The useful inter-relationship between the imaging technology utilized in this project and that which would facilitate the goals of project III-F, III-G, and eventually III-J (as well as several other projects under the direction of other PI's) are self evident.

Project 3I : "Complement C5a to Enhance Cell Uptake of DNA"
Principal Investigator: S. Ward Casscells, M.D.

In this project, there have been considerable expenditures ($311,781), mostly for the purchase of the BiaCore to permit the detailed structural biology studies required to examine the interactions of the membrane attack complex with the cell membrane. The remainder of the expenditures have been for studies aimed at developing improved reporters of gene expression and higher-resolution flourescence imaging to track the entry of transgenes into the cell and their transport to the nucleus. These basic studies have indeed gone well, as described for Projects 3F, 3G, 3H and 3L. These projects, the collaboration of Drs. Engler, Scott-Burden and Casscells, have improved our understanding of liposomal and peptide gene transfer and are progressing well enough that we have elected to focus on them and not attempt the C5a membrane-attack complex studies for gene transfer. Moreover the FGF-mediated transfer is nontoxic and indeed promotes cell viability, whereas the C5a method has some cost in cell lysis. It will therefore be retained as a back-up strategy in 1999.

Project III-J: "Mechanisms of FGF Transduction"

Principal Investigator: David A. Engler, Ph.D.

Year one progress has been limited to two of the four technical objectives of this project. We have successfully determined the phosphorylation specificity of pp90 and its subcellular localization pattern - post phosphorylation event (technical objectives 1 and 2). We have, as of yet, been unable to purify enough natural product that is free enough of contaminating compounds to yield usable N- and or C- terminal peptide data that would otherwise allow for its rapid cloning by PCR. Some experimental data (MALDI-TOF mass spectroscopy.) has been obtained that points to a molecular size for this protein that is considerably smaller than that identified by SDS-PAGE (reported in quarter 3). Until the time that this molecule can be unequivocally classified as to whether or not it is a previously unidentified molecule (i.e. whose gene has not previously been cloned) we will continue to devote our efforts to the remaining goals under technical objective 3 - the purification and sequencing/cloning of the pp90 gene. Once this has been accomplished, or we have gained enough information from peptide sequencing to assure ourselves of its identity by existing protein databases, we will embark on studies to further technical objective (4), that of trying to ascertain the functional role pp90 plays in FGF signal transduction throughout various cell types. It is presumed that this molecule will play significant key roles in directing the cellular response to signals elicited at the cell surface by FGF binding to its cognate receptors, the elucidation of which will provide opportunities to significantly impact outcomes of individual cellular scenarios by altering the types of molecular interactions pp90 is responsible for initiating.

PROJECT 3K: "Enhancing Resistance of Inhaled Toxins by Aerosol"

Principal Investigator: Dr. Henry Strobel

OBJECTIVE:

Cytochrome P450 in lung may be augmented to serve a more effective protective role against noxious agents in the environment or deliberately introduced into the air. The many forms of Cytochrome P450 present in the various tissues of the body endow individuals with a wide array of metabolic avenues to detoxify hamiful compounds. The lung is an excellent example of a tissue exhibiting the expression of many forms of Cytochrome P450. The lung is critical in that it is a major portal ot entry For toxic compounds into the body. Inhaled toxic compounds can be inactivated by various cytochromes P450 present naturally in the lung or transfected into lung cells by artificial means. The lung, therefore, is an important site for dealing with noxious agents for both of these reasons.

AIMS:

Our research is aimed at defining which cvtochromes P450 normally present in lung are able to detoxify noxious agents. Our approach involves using techniques of molecular biology to identify the individual P450 isoforms present and quantitate the amount of the forms present. This will enable us to know which forms could participate in detoxification of noxious agents. We will then define the metabolic routes used *in vitro* bv each of these candidate forms to detoxify chemicals. Having by these methods determined and defined the ability of natural lung cytochromes P450 to detoxify hazardous agents, we will devise systems to augment the naturally occurring P450 complement with other forms of P450 added exogenously to lung cells. Our proposal involves the encapsulation of CDNA vectors expressing single forms of P450 into simple lipid vesicles in an aerosol which can be inhaled. We believe we can target specific cells - lung macrophages - with these lipid vesicles. The use of macrophages as the target cell will provide a transient increase in Cytochrome P450 dependent metabolic capacity to resist an inhaled insult by noxious agents. This process would be an advantage to individuals subject to contaminated environments as well as those who work in areas with deliberately poisoned air.

PROGRESS:

Our progress this year can be summarized under three areas; definition of regulatory regions of the cytochrome P450 4F5 gene, transfection of genes into macrophages, and development of suitable assessment measures to define the presence and functionality of CYP P450 subfamily 4F in lung tissue and alveolar macrophages.

a) Our work on gene transfer to macrophages has enabled us to show expression of the CDNA for green fluorescent protein (gfp) in isolated alveolar macrophages. Using commercially available lipojfectin or lipofectamine allows the expression of gfp in I to I% of macrophages in culture. Using liposomes of stated composition allows a higher percentage transfection and expression. We will continue to optimize conditions for higher efficacy expression.

b) Substantial progress has been made on the partial characterization of the promoter and upstream regions of the gene for cytochrome P450 4F5 and on further definition of the catalytic capacities of purified cytochrome P450 4F5 recombinant protein expressed in E. coli. We have

studies underway to define in vitro parameters of cytochrome P450 expression and activities in rat lung and macrophages after treatment of animals with various drugs.

The promoter region of CYP 4F5 gene contains the non-traditional initiation site (CCAAT) and SPI binding sites seen in "housekeeping" genes and in some other P450 genes. CYP 4F5 is not induced by the peroxisomal proliferator clofibrate. In the upstream region we see only half sites for the PPAR binding site element consistent with the lack of induction by clofibrate. Retinoid binding sites are also seen in the upstream region as are NFκB sites associated with cytokine effects. All of these sites are consistent with the involvement of CYP 4F5 in aspects of the inflammatory response.

Purified P450 4F5 expressed recombinant protein has brisk activity toward chlorpromazine and lower activity with haloperidol, two drugs used in treatment of schizophrenia. Of more relevance to lung metabolism, P450 4F5 catalyzes the conversion of arachidonic acid to 20 hydroxy eicosatrienoic acid and other products. This metabolic activity is consistent with a role for cytochrome P450 4F5 and possibly other members of the 4F subfamily in response to modulators of the inflammatory response by either decreasing the amount/activity of the prompting modulators (leukotriene B4) or increasing arachidonic acid metabolism along an alternative pathway. In either case the 4F subfamily may be implicated in the lung's response to toxic chemicals.

C) We have also developed preliminary methods for adapting *in situ* hydbridization techniques for detection in control tissues, lung and alveolar macrophages. In this regard, we have developed a high affinity 300 base-pair probes - sense and antisense - to define the distribution of CYP 4F5 mRNA in tissues. In order to develop the technique and assess the probe, we used liver and kidney as control tissues because our previous results (Kawashima, Kusunose, Thompson and Strobel *Arch. Biochem. Biophys.* 347:148-154, 1997) have shown both that the CYP 4F subfamily is strongly expressed in these tissues and that its expression is down-regulated by clofibrate administration. Thus, these two tissues are ideal for definition of the efficacy of the probe and for determining appropriate conditions. Using this approach we have developed very sensitive probes for the 3'- and 5'- untranslated regions of CYP 4F5. When the antisense probe is used in liver, strong labeling is observed in the cortex region but not the medulla. When the sense probe is used very little to no staining is seen anywhere. In liver the use of sense and antisense probes confirms the presence of CYP 4F5 in liver but the distribution in liver is not regionally restricted. With these techniques in place we are now approaching the regional distribution of CYP 4F5 within lung tissue.

We are also developing methods to determine the distribution of CYP 4F5 in alveolar macrophages. In order to do so we must utilize our method for lung lavage to isolate cells and fix them to microscope slides. We will be able to do this with the aid of an instrument, which uses centrifugal force to funnel cells for deposit to a microscope slide. After drying and fixing, we will utilize our sense and antisense probes to define CYP 4F5 distribution.

PLANS:

In the coming year we will focus our efforts on definition of the expression of CYP 4F5 in alveolar macrophages, the characterization of the regulatory regions and promoters of CYP 4F5 and CYP 4F4, and a fuller definition of the range of catalytic activities of the CYP 4F subfamily.

PROJECT III L "GENE TRANSFER: MANIPULATING LIPOSOME STRUCTURE"

INVESTIGATOR Timothy Scott-Burden

1st quarterly report

Results: Several commercial liposomes preparations were investigated with respect to their ability to induce NO production. Western blot data confirmed that Lipofectamine strongly induced the expression of NO synthase2 (NOS2) and the concomitant expression of GTP cyclohydrolase. The latter enzyme is responsible for production of tetrahydro-biopterin, an essential cofactor for NO synthase activity. A number of different, commercially available liposome formulations were tested for their ability to induce NOS2 expression (leading to subsequent NO production) and they were found to vary widely in respect to gene induction. Since induction of NOS2 is a common occurrence in response to endotoxin, a contaminant in many commercial reagents, we tested for the presence of this reagent in preparations of liposomes. There was no evidence of lipopolysaccharide (endotoxin) in any of the reagents used in these studies. Also, induction of NO synthase gene expression by endotoxin followed very different kinetics to those observed for liposomal induction.

2nd quarterly report

Results: The kinetics of NO synthase induction by interleukin-1β, endotoxin and lipofectamine were compared in cultures of smooth muscle cells. Conditioned media samples were collected at timed intervals (see figure) and the levels of NO_2 ions accumulating in the medium used as a measure of NO production. Nitrite anion concentration was determined using the Greiss reagent. We have previously demonstrated that NO_2 levels in conditioned media from smooth muscle cells represents 75% of the total NO produced by the actions of NO synthase II (Scott-Burden, et al.1992). The rate of NO (NO_2) production elicited by liposomal treatment of smooth muscle cells was slower than that observed by cytokine treatment but considerably faster than that due to endotoxin. The latter has been shown to induce NO synthase by eliciting the production of cytokines and these then induce expression of the synthase. These data suggest that liposomal induction of NO synthase is mediated by a mechanism which is distinct from that associated with endotoxin action. In addition the data confirms our contention that liposomal induction of NO synthase is not due to contamination of the reagent by endotoxin.

3rd quarterly report

Results: Mock- and growth factor gene transduced smooth muscle cells, established in 12 well multiwell tissue culture plates were exposed to either cytokine or liposome solutions at concentrations shown to elicit maximal induction of NO production in previous investigations. NO production (measured as nitrite accumulation in conditioned media over 24 hr) was not significantly different for mock- and growth factor transduced smooth muscle cells. However, as already observed (see accompanying report for project III E) there were significant differences in the levels of NO produced by mock- and growth factor gene transduced cells that were exposed to IL-1β. These data are indicative of a divergence in the pathways leading to NO production in the cultured smooth muscle cells. Although the actions of FGF2 on NO production by smooth muscle cells exposed to IL-1β have been reported previously[1], the mechanisms involved have not been fully elucidated. Part of the actions of FGF2 relate to activation of an enzyme, GTP-cyclohydrolase, which controls cofactor (tetrahydrobiopterin) availability to NOS II. Data obtained on the synergistic effects of FGF2 and IL-1β suggested that the growth factor enhances the production of cofactor which is limiting when cells are exposed to IL-1β alone. If this is indeed the case it would appear that lipofectamine action leads to both induction of NOSII and elevated activity of GTP-cyclohydrolase.

4th quarterly report

Results: Tests of the Clontech materials have been completed and the various steps in the procedure learned and perfected. Preparation of total RNA (yields 700-500μg) have been completed from control, cytokine and lipofectamine treated cells. This will be in the assays described above.

Project 3M. "Electric Impedance and Near-Infrared for Localization of Plaque Lipid."

These two projects were conceived as alternatives to the infrared detection of inflammation. The electrical impedance idea was quickly dropped this year, when it was found to have been patented, investigated and dropped by a group several years ago. However, the near-infrared project has progressed exceedingly well. The near-infrared project has progressed from the conceptual stage to one in which each one of the hypotheses appears to be true—namely, 1) that inflammation produces changes in the near-IR spectrum; 2) that regional differences in pH and PO_2 can be measured in at least one model of inflammation (atherosclerotic plaque); and 3) that these differences are attributable, at least in part, to high concentrations of nitric oxide, hydrogen ion, reactive oxygen species, lipid and low concentrations of oxygen and glucose. This project has led to the design of a multiparameter catheter for identifying areas of inflammation, abscess, granulation and, potentially, malignancy. This project is detailed below.

Expenditures as of November 1, 1998, were $14,975.

PROJECT 3M

IR Spectroscopic Diagnosis of Atherosclerotic Plaques

1. INTRODUCTION

The rationale for this proposed research project is the well-recognized importance of developing new diagnostic devices for detection of vulnerable atherosclerotic plaques. These are the plaques at risk of rupture and/or thrombosis. These events are the ultimate trigger of most heart attacks and strokes. Plaque rupture triggers 60% to 70% of fatal myocardial infarctions (heart attacks) and many or most strokes.[147] It is now well recognized that monocyte macrophages contribute to rupture by releasing metalloproteinases, which can digest and degrade the collagens and other extracellular matrix components and, thereby, weaken the overlying fibrous cap. In a further 25% to 30% of fatal infarctions, the plaque does not rupture but becomes inflamed and eroded (e.g., the endothelial cells are lost).[147] Recent studies have shown that the risk of plaque rupture (its "vulnerability") depends more upon plaque composition than upon plaque size or stenosis (the degree of obstruction of blood flow).[148] Most ruptures occur in plaques containing a soft, lipid-rich core that is covered by a thin and inflamed fibrous cap. This cap is thought to rupture in response to stress (such as increased blood pressure or repetitive twisting and flexing, such that rapid heart rates statistically predispose to rupture), but spontaneous rupture due to proteolytic degradation or hemorrhage within the plaque have also been proposed.[147]

Other thromboses occur, not in response to rupture with exposure of the thrombogenic plaque contents, but rather in response to loss of the antithrombotic endothelial lining of the vessel or its conversion to a prothrombotic state by inflammatory cells and their products (which cause endothelial cells to express on their surface prothrombotic molecules).[149] The inflammatory cells themselves are also directly prothrombotic.[150]

The difficulty of determining which lesions are prone to disruption has been well documented, and this is a major reason that myocardial infarction and stroke are hard to predict.[150] Recent studies from our group have discovered that inflamed plaques give off more heat than do noninflamed plaques.[151] Inflammation has long been characterized by heat (calor, tumor, rubor and dolor being the classic features of inflammation), but local heat had not been described previously in atherosclerotic plaques. We found that the surface temperature of the cap surface is proportional to the density and metabolic activity of the inflammatory cells and to their proximity to the surface.[152]

We have also recently discovered a surprising degree of heterogeneity with respect to plaque pH. (described later in this proposal) Tissue pH is an important physiologic parameter that indicates both blood flow and the metabolic state of cells. Cell activation by growth factors or cytokines leads to slight acidification of the extracellular pH due to exchange of sodium for hydrogen. More marked acidification occurs in response to ischemia (inadequate blood flow to the tissue) because of the lack of oxygen. This impedes oxidative phosphorylation, and, in its place, synthesis of ATP becomes dependent upon an increase in anaerobic glycolysis. This results in accumulation of lactic acid. An inadequate synthesis of ATP and reduced wash-out of CO_2 and lactic acid also contribute to acidosis.

Examples of such environments include myocardial infarctions and abscesses (e.g., infected wounds). Our investigations have recently indicated that atherosclerotic lesions exhibit regions of low pH (e.g., 5.5 to 7.4) as well as foci of high pH (7.5 to 8.5). We hypothesize that acidic plaques are rich in activated macrophages and thus are prone to rupture or erosion. Testing this

hypothesis will require a nondestructive method of serially measuring plaque pH. Thus, we also hypothesize that Near-IR—which exhibits spectral shifts in response to pH alterations in other tissues—may be used to detect acidic plaques. Moreover, near-IR spectroscopic shifts have been described in other tissues accumulating NO and reactive oxygen species (recently described features of inflamed plaques).

Our third hypothesis is that near-IR spectroscopy will be useful in detecting these conditions in the living plaque. Fourthly, since near-IR can detect desaturated hemoglobin and is in widespread clinical use for this purpose, we propose that, if plaques have a low partial pressure of oxygen (due to increased O_2 consumption in inflammatory cells, as well as deficient blood supply), near-IR spectroscopy will also undergo characteristic shifts to reflect these conditions.

Our fifth hypothesis is that the recognized near-IR spectra for lipid will prove useful in detecting the large amounts of superficial lipid that predispose to plaque rupture or thrombosis.

Our sixth hypothesis is that near-IR spectroscopy will be useful in detecting regions of the plaque that are low in glucose. Such regions have not been described, but we predict their existence because of the recognized intense glucose dependency of macrophage metabolism.

Our seventh and final hypothesis is that a near-IR signature of some biocompatible tracer may be useful in early detection of certain types of vulnerable plaques with intraplaque hemorrhage or fissured cap.

Together, these conditions should combine to produce significant, characteristic and reliable shifts in the near-IR spectra. Combining this with the recognized shifts in near-IR and mid-IR spectra produced by temperature changes, we hypothesize that near-IR and mid-IR spectroscopy will have unique advantages in the nondestructive identification of vulnerable plaque and may eventually prove to be clinically useful.

Moreno et al have very recently reported that near-IR spectroscopy can distinguish normal arteries and stable plaques from inflamed, cholesterol-rich plaques in post mortem specimens of formalin-fixed aortic tissue from hypercholesterolemic rabbits.[153] This pioneering work raises the question of whether the differences are present *in vivo.* We hypothesize that the differences in vivo may be greater because fixation eliminates differences in temperature and O_2 and blunts differences in NO and oxidation state.

A) IR Spectroscopy Background

IR radiation is a form of electromagnetic radiation that lies within the range of 0.7 to 500 μm (wavelength). IR spectroscopy is a technique that studies the interaction between IR light radiation and matter. In biomedical applications, the "matter" is considered to be tissues and biofluids. The interaction between electromagnetic radiation and matter is that of energy transfer from the radiation to energy of matter, and vice versa. Theoretically, light radiation is considered as discrete quanta of energy called photons, the energy of which is proportional to the frequency of the radiation. Photons can be emitted or absorbed when an atom or molecule's energy states change. Spectral lines and bands are evidence of quantized energy states in matter and of quantized energy transfer between radiation and matter. The quantized states of molecules consist of electronic, vibrational, and rotational energy levels. For each electronic energy state of a given molecule, there are a number of possible vibration energy states, and for each of these, there are many possible rotational states. Generally, the energy involved in electronic transition is more than those of vibration or rotation and requires energies corresponding to that of ultraviolet or visible

radiation. The rotational and the vibrational transitions involve energies corresponding to that of IR or microwave radiation. Energy levels represent the current characteristic state of the molecule. The properties of the characteristic states are related to identity of the molecules, the structure of the molecules and the activity of any chemical processes that molecules undergo. When molecules absorb only one quantum of vibrational energy, the IR absorption bands occur in the region of 2.5 to 20 μm, and this is referred to as the fundamental, or mid-IR, region. Absorption bands induced by absorption of several vibrational quanta occur at higher frequencies, in the combination/overtone, or near-IR, region.

IR spectroscopy (often called vibrational spectroscopy) is a well established and widely applied technique in the analytical chemistry laboratory. It is commonly used to analyze chemical/biochemical compositions in a mixture. The fundamental principle of IR spectroscopy is based upon the fact that there are intra-molecular motions as a result of absorbing IR radiation. The absorption frequencies and intensities in the IR spectrum correspond directly to the chemical functional groups in the molecule, such as C-H, O-H, N-H, C-C groups. For example, the C-H stretching of methylene and methyl group (~3.4-3.5 μm) arises mainly from the cholesterol skeleton and the long acyl chains and other ester-containing compounds; amide I and amide II bands at ~6 μm and 6.5 μm, respectively, are due mainly to the protein content of the tissue. The biomedical application of IR spectroscopy is based upon the changes in tissue biochemical compositions that differentiate normal from diseased states. Many studies have confirmed that the changes of IR spectrum also depend upon variations in the chemical and physical environment such as pH, temperature, etc.

Near-IR spectroscopy covers the IR radiation range between 0.7 and 2.5 μm in wavelength. The technique has been used extensively for quantitative analysis and has become more widely used for the identification of organic/biochemical materials, that is, qualitative analysis. In combination with advanced data processing and fiber optic technologies, the technique has been applied to the biomedical field and demonstrates substantial potential for diagnosing diverse clinical diseases.[154-156] However, near-IR spectroscopy almost exclusively measures the overtones and combination bands of the chemical functional groups. Several large absorption bands (0.97, 1.4, 1.9 μm) of water, a common component in the human body, are examples of the NIR spectra. In biomedical applications, many clinical studies (especially in neonatology and pediatrics) have documented the ability of near IR for detection of changes in concentration of oxygenated hemoglobin (HbO_2), deoxygenated hemoglobin (Hb), and patterns of change in the redox status of cytochrome C oxidase (cytochrome a,a_3). The technique is rapid, accurate, operator-independent, and non-invasive.[157-162]

NIR spectroscopic analysis provides a rapid, multi-parameter, non-destructive and cost-effective diagnostic tool for clinical applications. It is also possible to develop non-invasive techniques of NIR detection. Diffuse reflectance spectroscopy, using NIR spectroscopy, can provide physiochemical information about opaque, blood-containing sites in the living body. The instrumentation can be constructed to be "user friendly" and can be automated, so that there are few operator-induced sources of error. However, no single technique can be perfect. near-IR spectroscopic analysis suffers from the lack of specificity to recognize the individual molecule band. It also requires a large data set to build the calibration model. The fact that near-IR theory lags behind experimental development has led to pitfalls for the inexperienced in deriving valid calibrations, and these pitfalls have delayed its acceptance as a useful analytical technique.

B) Application of IR spectroscopy technique to biomedical diagnosis

The application of near-IR spectroscopy to the clinical sciences has been developing for almost 10 years and has focused mainly on the pathophysiology, diagnosis and management of infants with head trauma or cerebral hypoxia. With the development of fiber optics and advanced computation and data evaluation techniques, biomedical application of near-IR spectroscopy is gradually being applied in other clinical fields, such as clinical chemistry, pulmonary and critical care, cardiology, plastic surgery and cancer.[163-168] This proposal focuses on the IR spectroscopic detection of vulnerable atherosclerotic plaques.

1. IR detection

IR spectroscopy is a versatile technique, and it is relatively easy to use. One advantage of IR spectroscopic analysis is that the measurements are not affected by the physical states of the sample: gaseous, liquid, homogeneous and inhomogeous solid samples can all be conveniently studied. In biochemical/biomedical analysis, minimal sample preparation and reagent-free assay are the most attractive factors for *ex vivo* investigation. IR spectroscopic detection methods include transmission, reflectance, micro-sampling measurements, and photoacoustic spectroscopy. Transmission measurements are used for analysis of biofluids. For the present research project, IR micro-sampling and fiber optical diffuse reflectance IR measurements are preferred.

Fiber optic technology accelerated the *in vivo* use of NIR spectroscopy. Due to the rapid advance of telecommunication development, optical fibers for delivery and transfer of NIR energy and information are readily available. Silica glass optic fibers work well through the visible spectrum and up to 2.5 μm of Near-IR. Fibers are available as clad, single fibers or as bundles of multiple fibers. Bundles may be randomized for optical uniformity and mixing, branched for multiple input/output applications, or constructed as spatially coherent arrays for imaging purposes. Using a fiber optics-based NIR spectroscopic diagnostic catheter, we can image the atherosclerotic plaque sites remotely.

IR microspectroscopy is a new technique that combines IR spectroscopy and optical microscopy.[169] Combined with a computerized movable stage, the technique allows for spectroscopic imaging of selected tissue sections in multiple wavelengths. In this way, biochemical structural information about the specimen can be obtained and directly correlated with tissue pathology/histology.

When near-IR spectroscopy was introduced to the clinical field a few years ago, clinical investigations using the new near-IR technology demonstrated that the technique had the potential to provide important qualitative and quantitative information. However, certain technical problems prevented widespread clinical use; among these were as fiber optic fractures and insufficient computational software capabilities. Although the spectrometer in use was factory produced, commercially available, and internationally distributed, the bioengineering support for the clinical studies was limited to the facilities and to clinicians. These factors restricted technical development and clinical applications.

2. IR-based multi-parameter diagnostic catheter

One of the specific aims of the present proposal is to develop a multi-parameter catheter for detecting vulnerable atherosclerotic plaques based upon visual and/or near-IR spectral analysis of successive sites along a vessel wall. A multi-parameter catheter assembly, which includes catheter, detector and data processor, will be used as an investigative tool to identify at-risk

plaques by obtaining near-IR and/or mid-IR spectral data for two or more physiopathologic parameters at successive sites along a vessel wall. The proposed research will provide an analytical system for establishing standard mid-IR or near-IR spectra for parameters associated with at-risk plaque. After receiving, processing and analyzing near-IR and/or mid-IR spectra obtained from the catheter, a predictive level of risk for plaques will be determined with the diagnostic algorithm.

A primary objective of the present proposal is to develop methodologies for more specifically identifying vulnerable (at-risk) plaques in a living patient by assessing the status of two or more chemical or biochemical parameters associated with vulnerable plaque. Another objective is to provide a method of predicting a level of risk of an atherosclerotic plaque by detecting, measuring, or quantifying two or more parameters associated with plaque that is at risk of rupture or thrombus formation.

A third objective is to provide a method of distinguishing dangerous plaque from normal vessel wall tissue and from relatively stable plaque. An important application that the present project may eventually provide is a method of detecting inflammation due to infection, cancer or autoimmune disease, or wound.

The method includes simultaneously measuring, at a site on a living vessel wall, two or more chemical parameters associated with activated inflammatory cells such as macrophages and T lymphocytes and smooth muscle cells. It is these highly active cells that are present in inflamed vulnerable atherosclerotic plaque. The qualitative or quantitative measurement of the following parameters will be performed: pH, nitrosyl hemoglobin, nitrosyl tyrosine, glucose, lactate, oxidized collagen, oxyhemoglobin, reduced hemoglobin, oxidized cytochrome oxidase aa_3. In addition, by using a dye that shifts the near-IR spectrum, it may be possible to detect leakage of dye into a plaque, whereby a plaque cap fissure is revealed. The detection is accomplished by simultaneously measuring in a living vessel at least two chemical parameters, factors, or analytes that are associated with inflamed, vulnerable atherosclerotic plaque. This includes providing a fiber optic catheter having an illumination fiber bundle and a detection fiber bundle capable of directing light onto, or receiving light from, a site on a vessel wall. The catheter incorporates accessories for reducing optical interference by blood or other fluid within a vessel when undergoing examination. It also includes providing a source of 400-2500 nm wavelength light and a spectrometer, each operatively linked to the corresponding fiber bundle.

3. Data analysis and algorithm development

A multivariate regression technique will be used to identify subtle changes in the collected near-IR spectra, related to the analytes of interest that may not be noticeable by visual inspection. To perform the multivariate regression, a calibration data matrix R of spectral responses at selected wavelengths is first generated from a given set of calibration samples. The calibration data matrix R is then assumed to relate to a matrix of concentration values C by a model equation such as:

$$C = RS + E,$$

where S is a matrix of regression coefficients determined by the least squares method, and E is the residual error. The matrix of concentration values C is determined by an independent method of measuring analyte concentrations in the calibration samples. This model is the basis of the multiple linear regression (MLR) method. Other model equations may be employed, such as:

$$C = (RV)S + E,$$

where V is a matrix of eigenvectors of R chosen to "score" the indications of the analytes in a chosen eigenspace. This model is the basis of the principle component regression (PCR) method. The PCR method may be extended in a partial least squares (PLS) modeling procedure so that the underlying factors of both R and C are simultaneously estimated in an effort to provide better predictive success. Each of these methods may employ a classical least squares cost function, thereby seeking to minimize the sum of the squares of the residual error E.

To identify a preferred method or model, a predictive residual error sum of squares (PRESS) procedure may be employed. In this procedure, two sets of calibration samples are employed. The following steps are employed:

The first set (called the training set) is used to identify a regression coefficient matrix S. The second set is analyzed by the model to determine a matrix of estimated (predicted) concentration coefficients C. The estimated concentration coefficients matrix is compared with the independently measured concentration coefficients. The method which yields the smallest sum of squares of the errors between the predicted and measured concentration coefficients is selected as the preferred method.

More details regarding these methods and the selection procedure can be found in standard reference texts.[170-172]
Once a model has been chosen and a matrix of regression coefficients S has been identified, an analyte concentration coefficient vector c may be calculated by signal processor 127 from a spectral frequency measurement vector r:

$c = rS$ (for the MLR method), or
$c = r(VS)$ (for the PCR method).

2. PRELIMINARY STUDIES

Infrared spectroscopy is a versatile technique used in molecular functional group characterization. Although widely used in analytical chemistry for many years, its use in biomedical and clinical studies is new. In biology, IR spectroscopy is used to provide information about protein folding and the structure of proteins in solution. A few studies of clinical applications have been done, including blood oximetery, non-invasive glucose monitoring and neural function mapping. Our research group has obtained some preliminary results of IR spectroscopic imaging of the vulnerable atherosclerotic plaques from human carotid endarterectomy specimens. The near-IR spectra of the freshly harvested plaque samples were measured from 400–2500 nm. We also attempted to monitor certain tissue physiological parameters (i.e., temperature, pH, etc.) using near-IR spectroscopy.

A) IR spectroscopic imaging of atherosclerotic plaques

As normal tissue becomes diseased, biochemical changes occur, some of which alter tissue IR spectra. The IR spectra of human tissues are complex. There is considerable overlap of absorption features from the major molecular species present. An understanding of tissues is necessary to correctly assign the absorption bands. Although cellular and biochemical composition vary from tissue to tissue, certain constituents are common to all human tissues. These include lipids, protein nucleic acids, and carbohydrates. Theoretically, the IR spectra of most tissues should be closely approximated by the summation of these common constituents. Because specialized tissues may contain species that are not found in other tissues, investigators need a

precise understanding of the histology, pathology, and biochemistry of the tissue under investigation to accurately interpret the spectroscopic results.

Atherosclerotic plaque within a vessel results from a chronic, continuous process involving lipid infiltration into the intima, endothelial injury, leukocyte migration into the subendothelial area, continuous lipid accumulation inside the macrophages, endothelial thickening, and smooth muscle cell proliferation. This is followed by increased matrix protein and collagen production, and development of a fibr optic cap. In some cases, usually in the advanced stage, hemorrhage and calcium deposition occur, which increase the plaque's mass and cause it to protrude eccentrically toward the endoluminal space and narrows the lumen of the artery. When a plaque progresses to a vulnerable plaque, it is characterized largely by the presence of active macrophages, a free lipid core, a thin fibrous cap, and sometimes endothelial denudation and microthrombi.

Fig. 33 is a histology picture with traditional hematoxlyn and eosin stain. Fig. 34 shows visible imaging and the IR spectroscopic imaging of a human carotid atherosclerotic plaque thin section. The sample was collected during a carotid endarterectomy operation and immediately frozen with liquid nitrogen. The frozen sample was cut to 5 μm thin sections. One advantage of IR spectroscopic imaging is that tissue staining is not required. The IR spectroscopic imaging was made with an EQUINOX 55 FT-IR spectrometer (Bruker, Germany), and an IR microscope attachment was used to perform the IR tissue spectroscopic mapping. A 15μIR objective was used for the mapping. The sliced tissue section was mapped over an area of 9750X3750 μm. The map had a spatial resolution of 250 μm and consisted of 585 individual spectra. We used an integrated band area of C=O stretching vibration at 5.7-5.8 μm as the mapping parameter for the distribution of the lipids. This is shown in Figures 34A and 34B. The image of the tissue section's protein distribution, shown in Figures 34C and 34D, was generated by using an integrated band area of amide I 5.9-6.1 μm, which arises from the C=O stretching vibration that is weakly coupled to the C-N stretching vibration. These figures show that colored contour map and height of the peak correspond to the distribution and content of biochemical components. Athough the spatial resolution of the IR spectroscopic image was not high, our preliminary study of the histochemical analysis of thin plaque sections was able to show the distribution of biochemical compositions within the plaque. A further examination of specimens with high spatial resolution will make it possible to make more detailed IR spectroscopic images of atherosclerotic plaque in various pathologic conditions.

B) Near-IR spectroscopic measurement of atherosclerotic plaque in living tissue

Near-IR spectroscopy has been used for many years in continuous, non-invasive, bedside monitoring of blood oxygen saturation. The technique is based upon relatively simple principles. First, each molecule existing in the real world has its own characteristic and unique absorption spectrum. For example, water and hemoglobin absorb at different wavelengths in Near-IR. Second, the amount of absorption is proportional to the concentration of the substances in the sample. However, near-IR spectrometry is also associated with low molar absorptivities and overlapping absorption bands, and has a strong tendency to scatter in physiological samples. It has, therefore, long been considered inappropriate for analytical research.

With the advent of the semiconductor charge-couple device (CCD) photo detectors and advanced data processing techniques, it is now possible to collect and analyze a large number of data sets in a short period of time. In addition, new instrumentation and data analysis algorithms have been developed, and researchers devise new methodologies almost daily. The goal of our proposed research project is to develop an *in vivo* methodology for diagnosing atherosclerotic plaques in various stages using near-IR spectrometry.

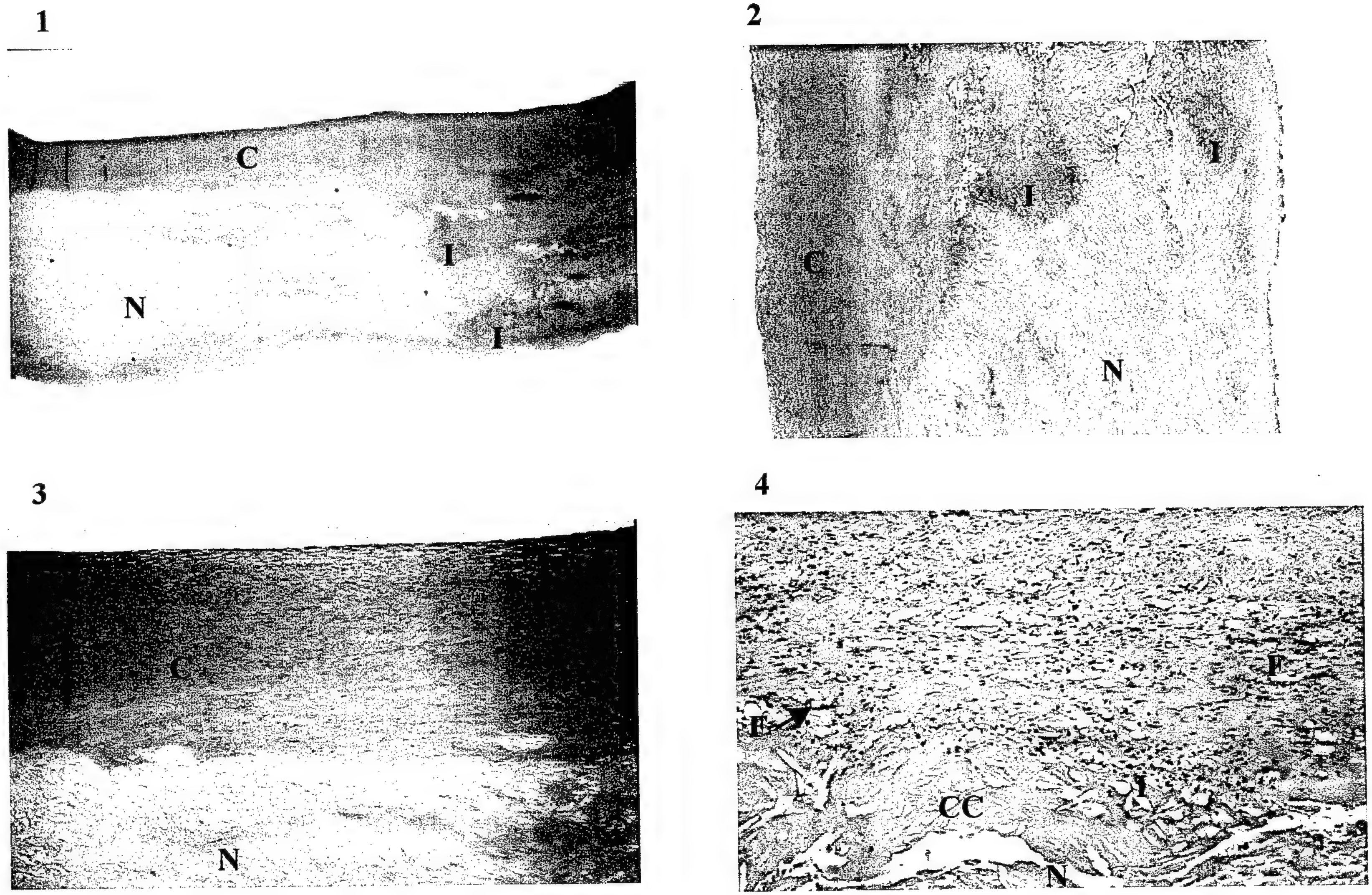


Fig. 33 Microscopic examination of thin section (5μm) of atherosclerotic plaque. A sharply demarcated, irregular,central region of acelluar lipid necrosis (N) is covered by a thick, intact, dense, hypocellular fibrous connective tissue cap (C). At one lateral margin, the necrotic core is marginated by a moderately discrete, irregular band of foam cells (F) admixed with small extracellular cholesterol clefts (CC) numbers of mononuclear inflammatory cells (I). The cap measures less than 0.1 cm in thickness. The lipid core measures up to 0.25 cm in thickness. Original magnifications: 1 (4×), 2 (10×), 3 (25×), 4 (40×).

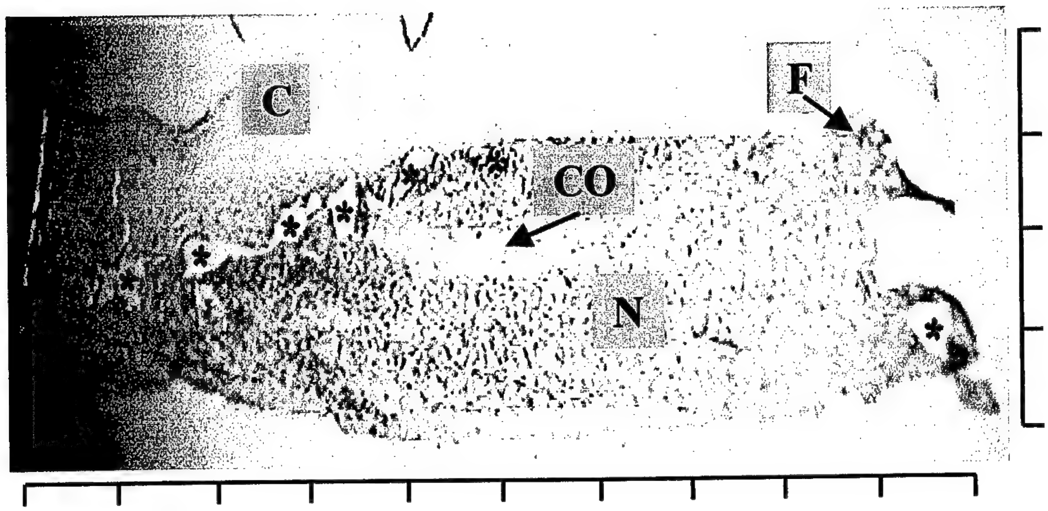


Fig. 34 Visible image of a frozen human carotid atherosclerotic plaque by CCD camera. The fibrous cap (C) corresponds to the blue-green edge of figure 2A, and figure 2B, indicating a protein-rich area. In figure 2C, F denotes one of several lipid-rich regions of foam cells. The necrotic core (N) is a mix of lipid, (yellow area in Fig. 2A) and collagen (CO). The asterisks (*) denote tissue-free artifacts of sectioning.

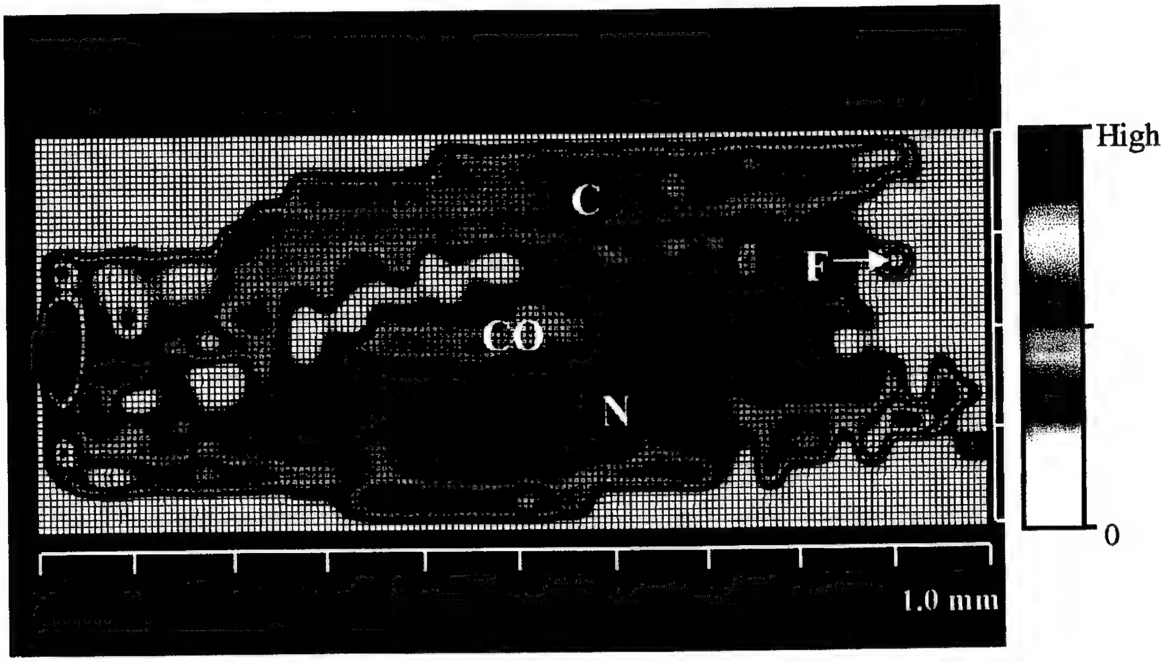


Fig.34A Two-dimensional MIR spectroscopic image of protein distribution, imaging parameter: integrated band area of Amide I absorption 6.1-5.9 μm. C denotes the protein-rich fibrous cap. F denotes foam cells. N denotes the necrotic core that contains only collagen-rich region (CO). The red region at the left is an artifact due to wrinkling of the section, making it thicker so that the signal is increased.

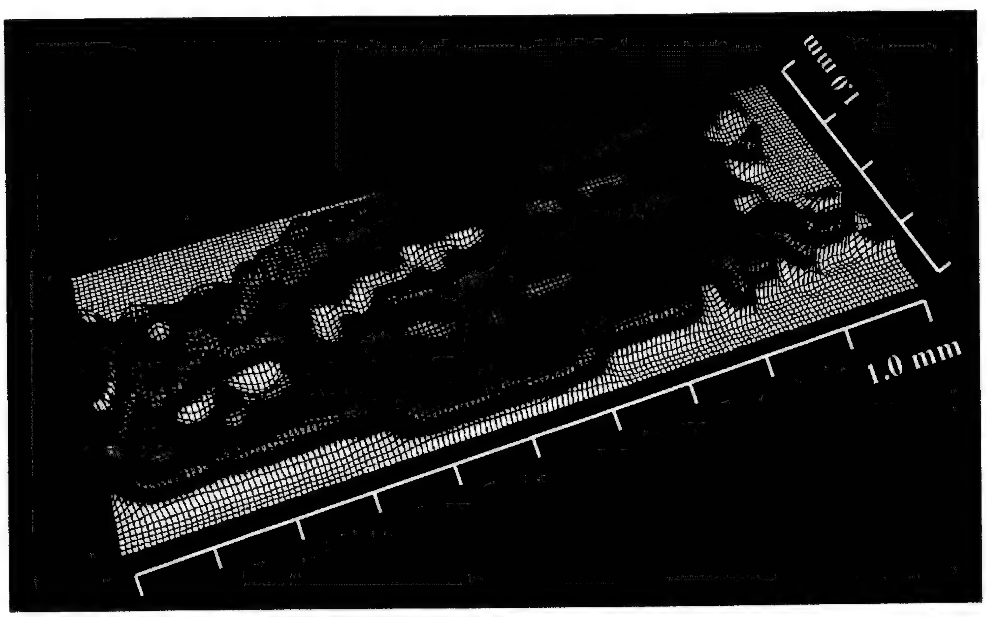


Fig.34B Three-dimensional MIR spectroscopic image of protein distribution using the same imaging parameter

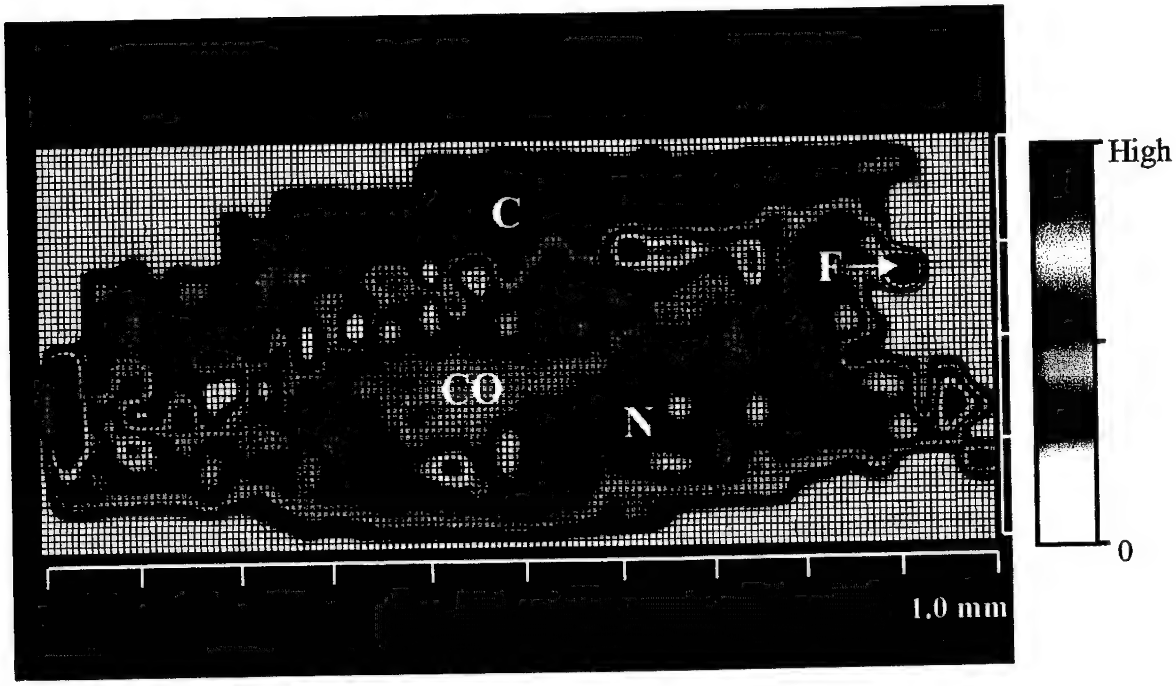


Fig.34C Two-dimensional MIR spectroscopic image of lipid distribution, imaging parameter: integrated band area between 5.7-5.8 μm. F denotes lipid-rich foam cells, yellow and red region of the necrotic core (N) reflect the presence of intracellular and extracellular cholesterol and esterified .cholesterol. The cap (C) and a linear region of collagen (CO) in the core, are blue (lipid-poor).

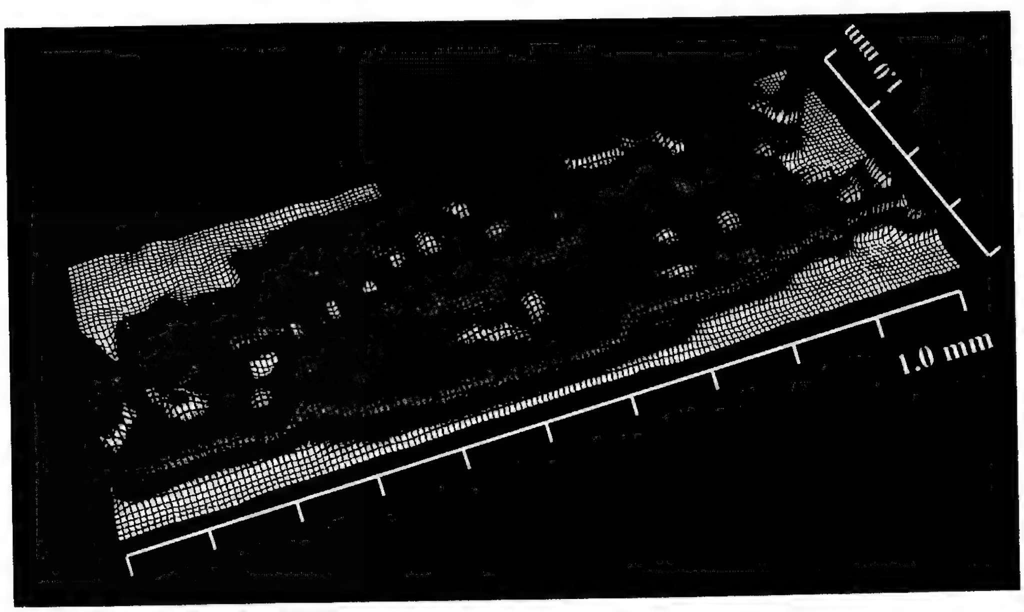


Fig.34D Three-dimensional MIR spectroscopic image of lipid distribution using the same imaging parameter.

An attractive feature of near-IR spectroscopy is its *in vivo* applications due to the relatively deeper penetration in tissue as compared to Mid IR. As discussed earlier, the major absorption feature in the NIR region is largely a result of the X-H functional groups (X = C, O, N, etc.). In this region, the intensities of the absorption bands of these functional groups are 10 times less than the intensities of these groups in the mid-IR region. However, the optical properties of IR radiation in the near region allow us to determine the deeper biochemical changes that occur as the plaque develops. To identify the near-IR signature of atherosclerotic plaque, a preliminary study of plaque was performed ex vivo in fresh tissue, and near-IR diffuse reflectance measurements were made. Human carotid atherosclerotic plaque containing normal tissue was collected during a carotid endarterectomy operation. The specimen was immediately transported to the laboratory for near-IR analysis. Fiber optic probe attached to NIRS 6500 near-infrared spectrometer (FOSS NIRSYSTEM, Silverspring, MD) was used to collect the spectrum in the wavelength region of 400 – 2500 nm.). Spectra were obtained from both atherosclerotic and normal parts of the sample. Thirty-two scans were averaged to obtain a single spectrum. Measurement of each spectrum required less than one minute. Absorption spectra of normal and diseased tissue did not show obvious differences (Fig. 35). However, second derivative of absorption spectra demonstrated striking differences between the two parts of the tissue (Fig. 35A, B, C). This preliminary data show that atherosclerotic tissue can be distinguished from normal tissue by near-IR spectroscopy.

C) IR/near-IR spectroscopic correlation of temperature in biological specimens

Elevated temperatures are associated with infections, wounds, cancer, autoimmune diseases, and atherosclerotic plaques. We have studied 61 atherosclerotic plaques removed from 61 patients during carotid endarterectomy performed for symptoms or for severe angiographic stenosis. Using a 24-gauge needle thermistor (Cole-Palmer Model 8402-20) in a 37°C chamber, the specimens were examined at 2-mm intervals, yielding approximately 30 measurements per plaque. These measurements established the background temperature. Typically, 6-7 zones per plaque were warmer or cooler than the background temperature by more than 0.2°C.

Near-IR spectrometry of water absorption is highly temperature-dependent. Because water constitutes more than 60% of human body weight, our preliminary studies were initiated to determine the absorbance of water at different temperatures. A fiber optic probe attached to NIRS 6500 near-infrared spectrometer (FOSS NIRSYSTEM, Silverspring, MD) was used to collect the spectrum in the wavelength region of 400-1700 nm.) Deionized water was kept at constant temperature of 28-53°C. Fig. 36 shows the absorbance spectrum of water. Changes in water temperature reflect changes in absorbance. Thus, the tissue temperature can be determined by measuring absorbance of water in the tissue. We will measure the temperature of the vulnerable atherosclerotic plaques using near-IR spectrometry in the proposed study.

D) IR/near-IR spectroscopic correlation of pH in biological systems

1. pH Heterogeneity of Atherosclerotic Plaque

We have recently hypothesized that different regions of atherosclerotic plaques may exhibit different pH value or variable hydrogen buffering capacity. Our major rationale for this hypothesis is as follows:

a. Chronic Active Inflammation: Inflammation plays an active role in both the development of atherosclerotic plaques and its rupture.[173] Increased metabolic activity of inflammatory cells (mainly because of anaerobic metabolism) leads to increased macrophage lactate efflux, thereby decreasing extracellular pH. Oxidized LDL has been shown to aggressively stimulate activity of macrophages and lower their environmental pH.[174-177]

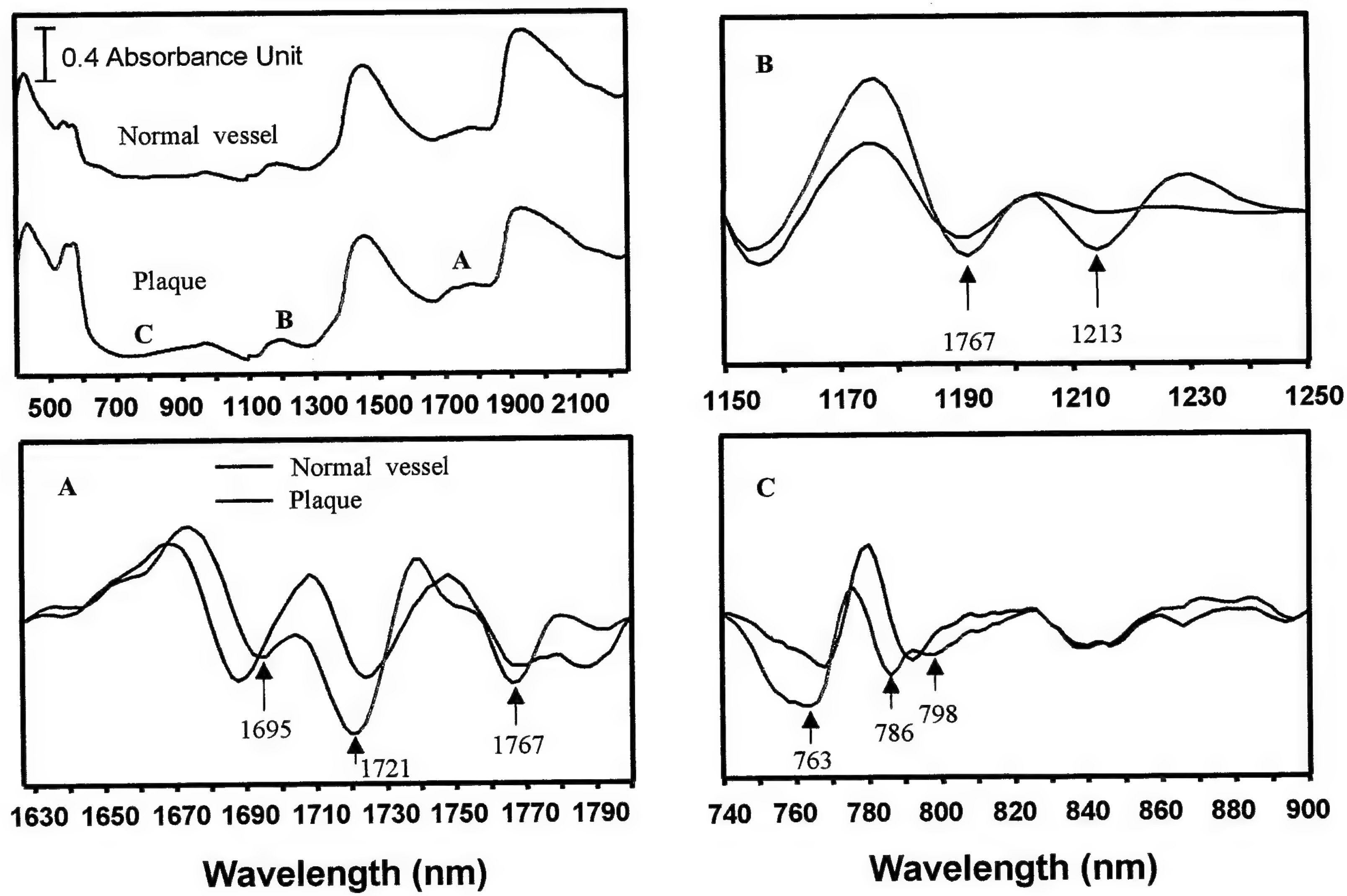


Fig. 35 NIR spectra of human atherosclerotic plaque and normal vessel. The A, B, an d C are the second derivative of the original NIR spectra in the regions between 1630-1800, 1150-1250, and 740-900 nm in wavelength. Spectra changes are virtually observable. Trace A and B are the C-H stretching first and second overtones respectively. The major absorption features between 740-900 are related to change of tissue oxygenation.

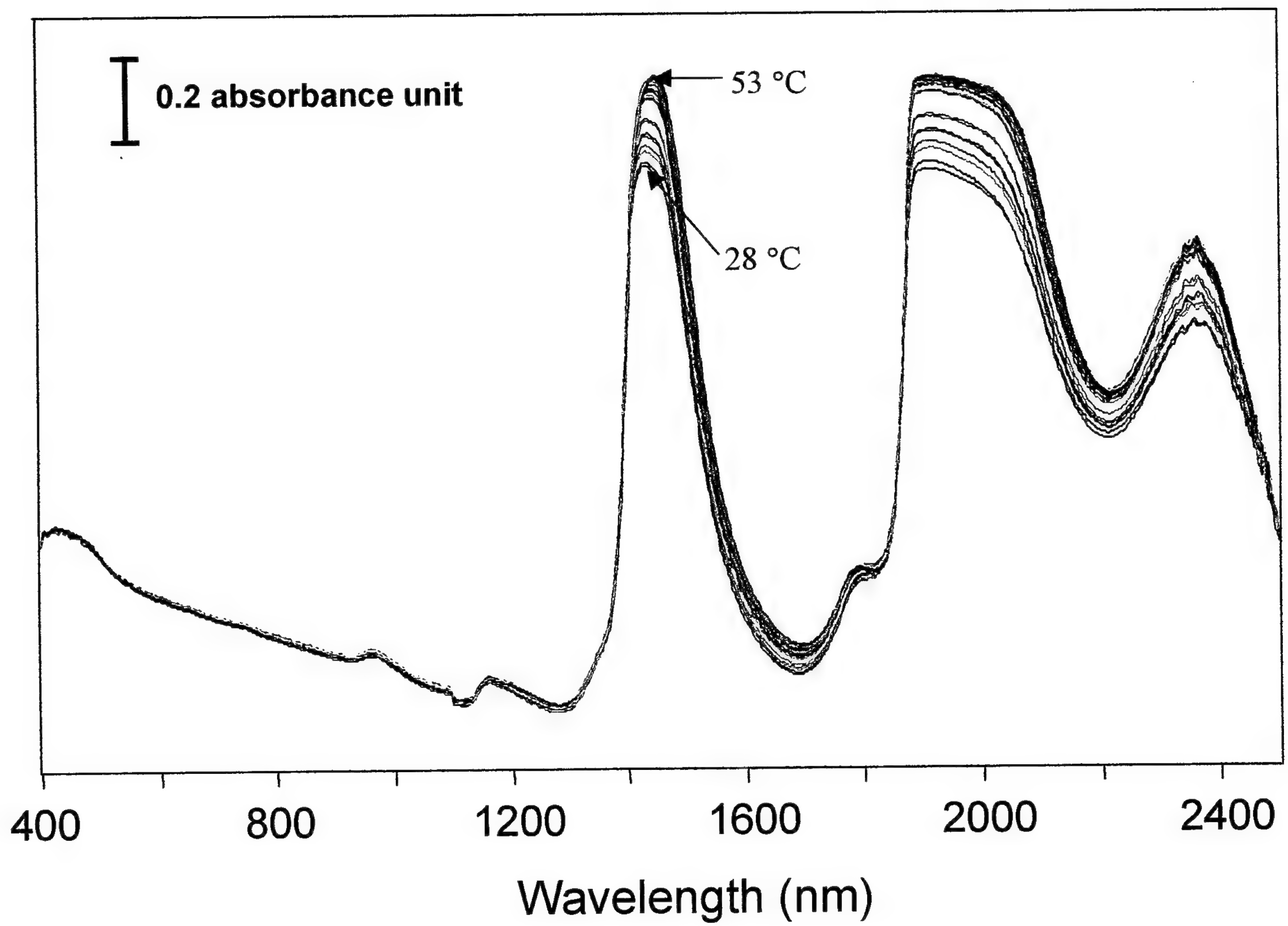


Fig.36 Near infrared transmittance spectra, pathlength ~ 1.3 mm, of water in various temperature ranging from 28 to 53 °C

b. Hypoxia: Growth of atherosclerotic plaque may introduce the plaque core to hypoxia due to limited oxygen permeability and consumption of O_2 by cells closer to the lumen through the cap, especially if the thickness of the cap exceeds 0.2 mm. Oxygen content of atherosclerotic plaque has been documented to be lower than normal in vascular tissue.[178-179] Thus, an atherosclerotic plaque may be exposed to a chronic anaerobic status leading to a low pH, or in a long term, to a low pH buffering capacity. However, angiogenesis in plaques, which can be triggered by hypoxia, may improve oxygen delivery. Since plaque angiogenesis is variable, this increases the chance that some plaques are hypoxic.

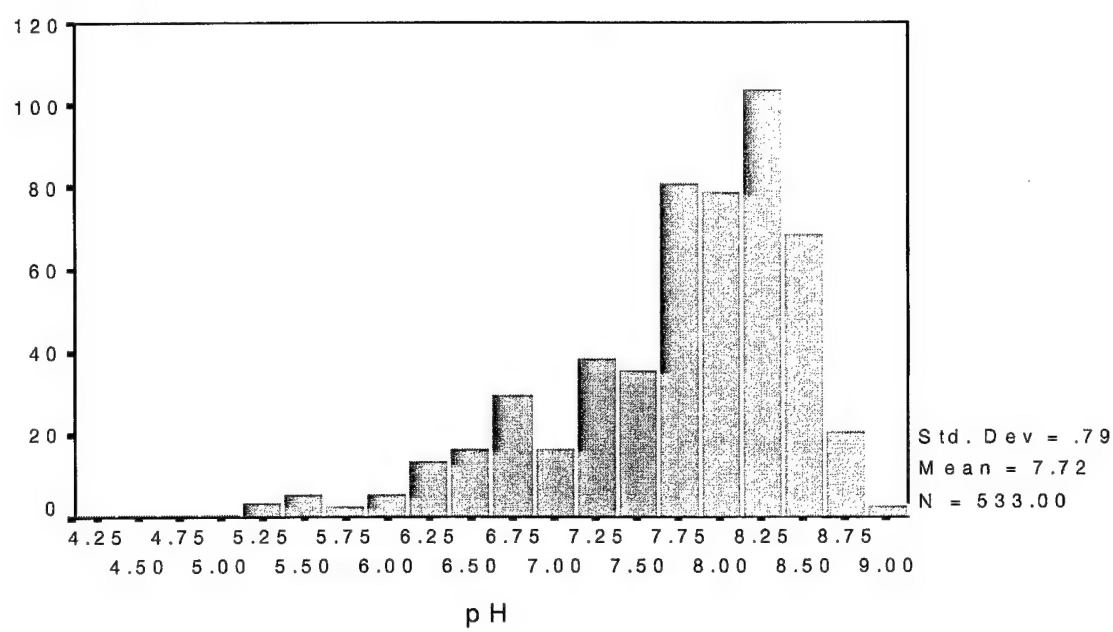
c. Calcification: Some atherosclerotic plaques show extensive calcification, and others do not. Some plaques even undergo ossification.[180] It is well known that calcification (calcium phosphate deposition) and ossification occur in a region of low pH with high calcium and phosphate.[181] On the other hand, calcium salts should be alkaline. If so, plaques should exhibit areas of low pH and high pH.

2. Measurement of Atherosclerotic plaques pH

a. Microelectrodes

We have employed a 21-gauge standard glass type pH microelectrode (Microelectrodes Inc., Bedford, NH) and Orion pH meter-750 to measure pH of human fresh carotid endarterectomized atherosclerotic plaques immediately after surgery. The accuracy of the system is 0.1 and sensitivity is 0.02. pH of plaques were measured at average of 0.2 mm depth from the surface of the plaque using a micromanipulator. All the measurements were done in incubator with a temperature 37°C. In order to correlate pH and histopathologic characteristics of plaque, we have used five different dies to mark different ranges of pH. Fig. 37 shows our preliminary results of pH measurement in 21 living carotid plaques from 21 patients. As clearly shown, there is marked pH heterogeneity

Figure 37. pH heterogeneity in 21 living human atherosclerotic plaques, measured by microelectrode.



We have measured pH of about 20 points in each plaque. Fig. 38 shows a marked within and between plaque pH heterogeneity.

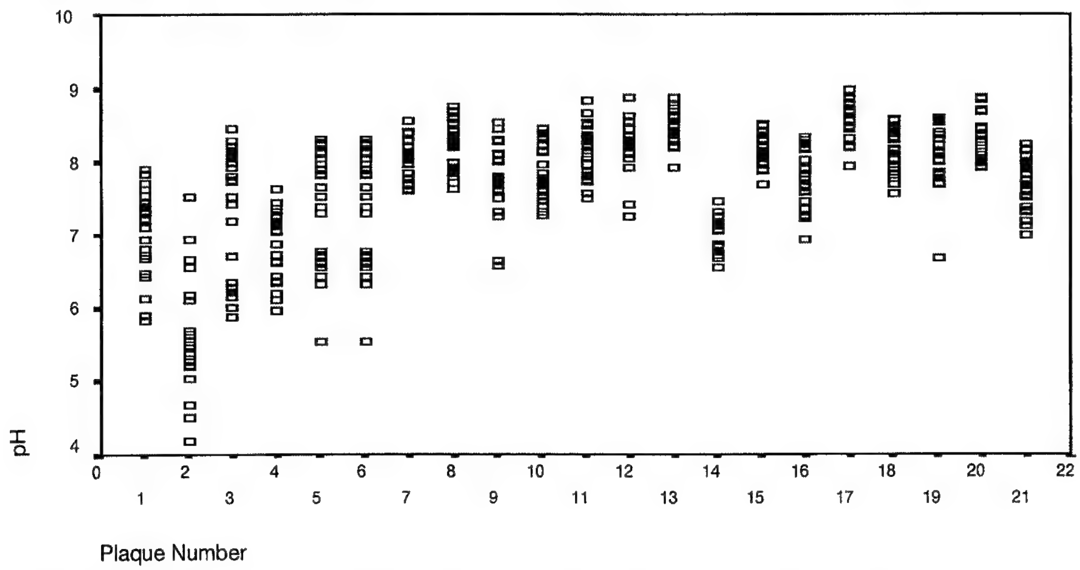


Figure 38. Showing a marked within and between plaque pH heterogeneity.

Macroscopically different plaques (yellow, calcified, and ruptured with thrombosis) showed significant variance of pH (photographs of plaques with pH measurement, Fig. 39).

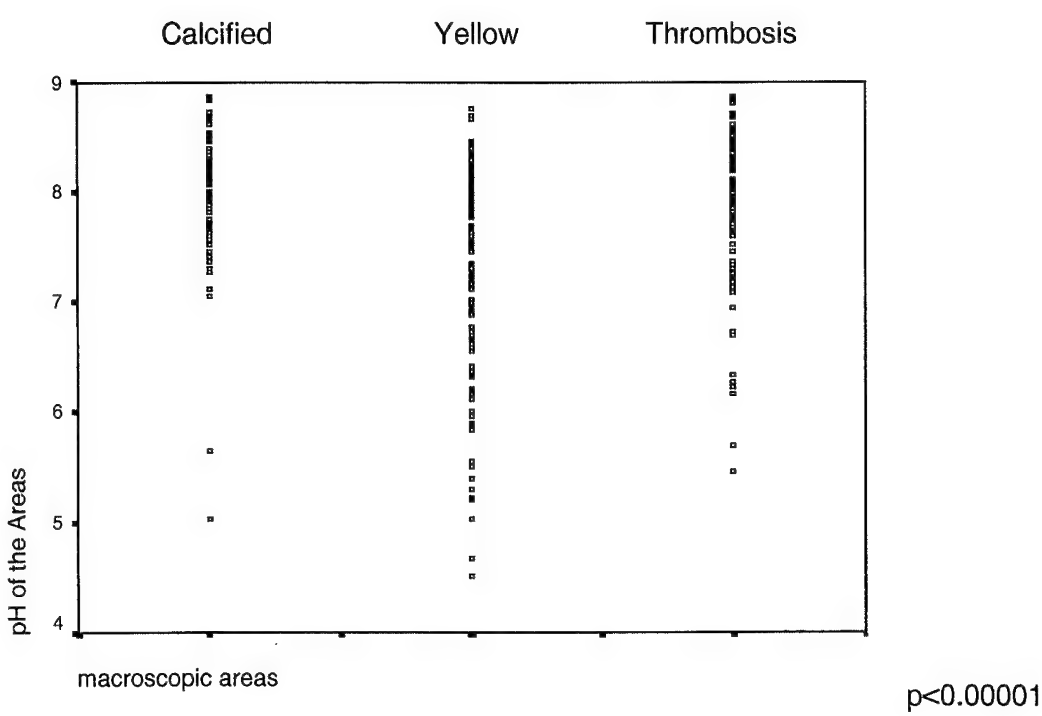


Figure 39. pH heterogeneity varies by underlying histopathology

As mentioned before, areas with marked calcification exhibit alkaline pH. We also found that most of the thrombotic areas were superimposed on calcified regions. Most regions were alkaline. The areas of low pH were mainly areas of inflammation.

pH Heterogeneity in Hypercholesterolemic Watanabe Rabbit

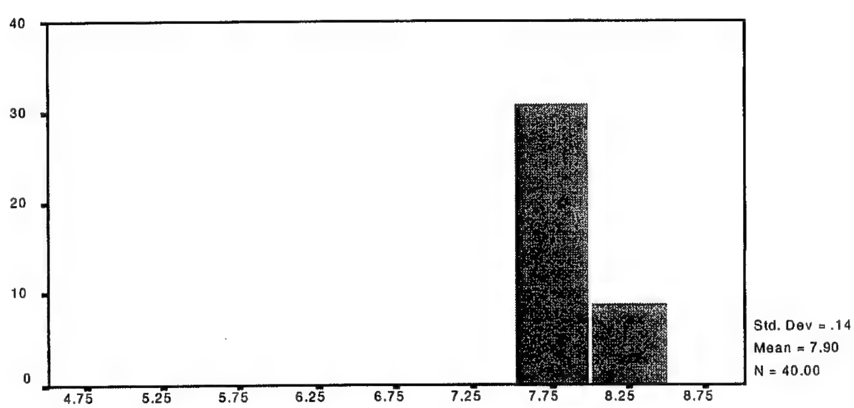
We have done similar microelectrode pH measurement of normal as well as atherosclerotic regions of rabbit aorta. As shown in Fig. 40a and 40b, normal aorta had little variance in pH, while atherosclerotic regions exhibited considerable heterogeneity and more acidic areas than did normal aorta.

This may lend further support to our hypothesis that atherosclerotic plaques, depending upon their stage of development, show different values of pH.

Fluorescence Ratio Imaging Microscopy (FRIM)

To validate the microelectrode, we demonstrated pH heterogeneity with pH paper, but diffusion and varying degrees of plaque water limited its accuracy. Fortunately, the development of sensitive fluoroprobes permits measurement of Ca^{2+}, cAMP, NO, Na and pH. The fluorescein derivative 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein [BCECF] estimates intracellular and extracellular pH (Martin and Jain, 1994). FRIM involves ratioing the emission intensity of BCECF (at 528nm) from dual excitation at 495nm (where excitation efficiency increases as pH increases from 6 to 8) and at 440 nm (where pH has no effect). A ratioing of these emission intensities (see below) has a number of important advantages: it eliminates inconsistencies due to photobleaching of the probe with time and problems due to variable sample thickness and nonuniform probe distribution. FRIM also reduces variables such as probe concentration, detection efficiency, as well as excitation pathlength differences.

$$\text{Ratio (R)} = \frac{\text{495 nm fluorescence intensity- 495 nm background}}{\text{440 nm fluorescence intensity-44 nm background}}$$



Normal Rabbit Aorta

Figure 40 a.

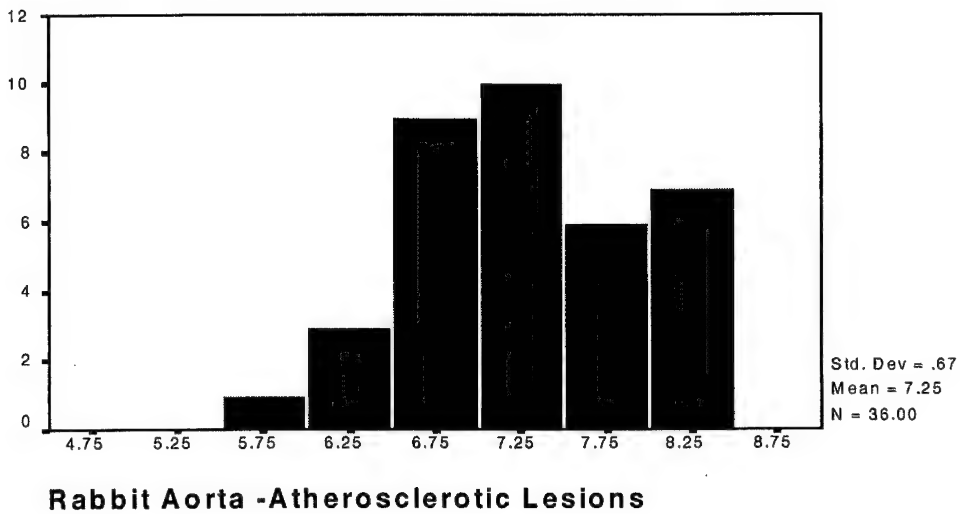


Figure 40b. pH heterogeneity in aortic plaques in cholesterol-fed Watanabe heritable hypercholesterolemic rabbits. Specimens were studied in the living state.

For detection of pH heterogeneity in vascular plaques we have used 20-40μm cryostat sections across the lumen of the vessel and plaque. Unlike individual cells imaged for pH by FRIM[182], the *in situ* plaque is simply too thick to image using fluorescence technology. pH values have been successfully estimated in brain cryosections in a similar fashion[183]. Our preliminary observations indicate that the freezing technique does not appear to adversely affect the pH heterogeneity across the plaque as determined by our microelectrode detection method (Figure 9). We shall continue to use this procedure, as it will enable us to examine larger plaque characteristics as well. The procedure will involve embedding of serial pieces of plaque, freezing them and then obtaining cross-sections of entire plaque thickness. An entire rendition of a plaque will be possible through use of Bitplane (Zurich) 3-D volume rendering software that we currently employ for rendering of multicomponent cytoskeletal networks in tissue culture cells (VanWinkle, et al., 1999, submitted). For additional experiments during the course of the proposed grant, we shall continue to use the FRIM technique employing a specialized microscope system currently marketed by Wallace/Olympus America Concord. This is a digital imaging fluorescence microscope/computer system with many capabilities: pH measurements, membrane capacitance, and calcium quantification. Especially useful for our pH ratiometric studies of vessel plaque pH heterogeneity is the Spectramaster multiwavelength controller built into the system. This permits us to write in the above ratiometric equation as well as to incorporate the pH calibration curve (see below) in such a manner as to generate true pH measurements at selected regions of the ratioed image.

For pH calibration, we shall use an adaptation of Martin and Jain's[184] procedure. Our calibration will involve use of a solid agar gel from which cylindrical plugs are cut. A plug is then frozen for cryostat sectioning at 100μm. The circular section on a glass slide will be incubated in a small drop of phosphate buffer (monobasic/dibasic) at each of 12 different pH's values from 5.0 to 9.0. pH of the circular gel slice will be monitored by micro pH electrode. After removing excess buffer by blotting, a drop of BCECF (0.02mg/ml) will be placed on the circular gel for 30 sec prior to blotting excess probe. Ratioing (as described above) will then determine the ratio (R) at each pH and will be plotted by computer program in the microscope system. A color bar indicating color mating with individual pH[185] will be included at the left margin of each imaged section.

Determination of pH values will be carried out at selected regions of the generated microscopic ratioed images and precise numerical pH values included at each circled region of the photograph. The BCECF form which we have used in our preliminary studies detects pH in the interstitium. Should we desire to plot intracellular pH, we shall use the ester form, BCECF-AM, an intracellular esterase-cleavable pH probe (Fig. 41)

Fig. 42 shows the cross section of entire vessel plaque with lumen (light blue) at top and heavy calcified region (dark blue-black) at bottom. This radiometric image (dual excitation at 495 and 440nm, single emission at 528nm) indicates a heterogeneous pH distribution across the plaque with scattered alkaline region prominently depicted in red. Subsequent assessment of selected regions and plotting into calibrated pH ration curve will give precise pH values in selected areas.

pH and near-IR spectroscopy

It has been reported that changes in tissue pH cause changes in near-IR spectrum.[186-187] We hypothesized that pH of atherosclerotic plaque can be determined based upon absorbance spectra of the hemoglobin histidyl rings in the physiological pH range of 6.0 to 8.0 It is not known yet whether NIR spectroscopy can reliably estimate pH of atherosclerotic plaque. Since the plaque is a very inhomogenous tissue it might be even more difficult.

pH and Plaque Rupture: Marker or Mechanism?

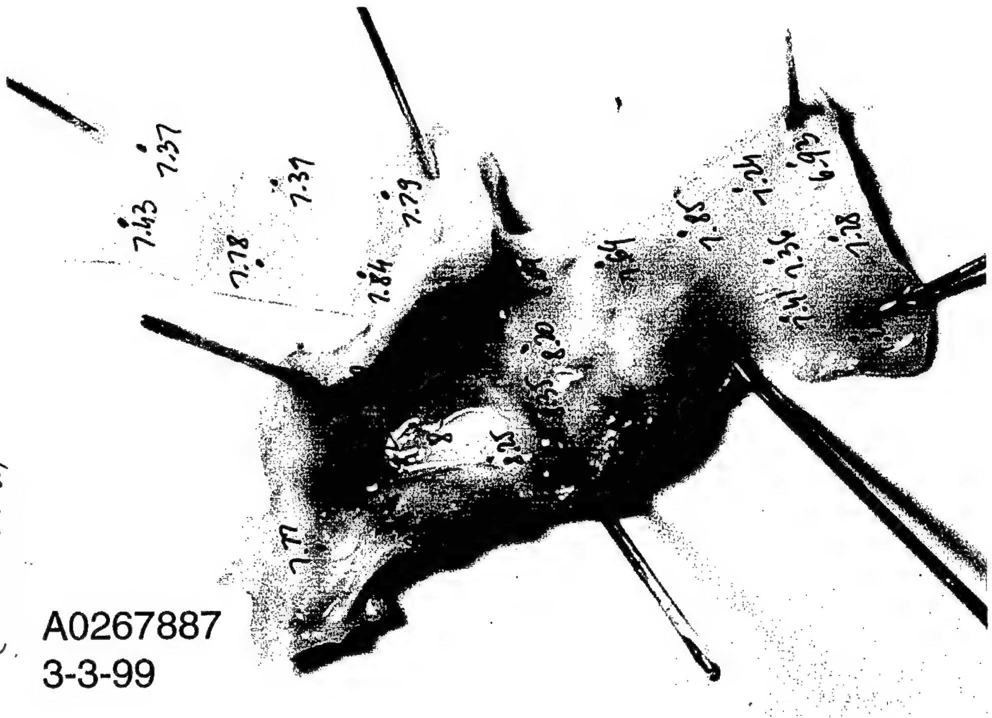
We hypothesize that acidic regions in the plaque are regions that are rich in inflammatory cells and thus regions at risk of rupture. The closer these regions are to the intimal surface where the catheter will be making the measurements, the stronger the signal will be. Thus, a strong signal will correlate with proximity of the inflammatory cells to the surface and thus will be proportional to the risk of erosion or rupture.

Another reason to hypothesize that pH will predict plaque progression and cardiovascular events is that an acid pH may be not only a marker but an exacerbating pathophysiologic variable. Two mechanisms can be hypothesized. The first is that acidic pH has been reported to reduce cysteine-and-histidine-based antioxidant function, as well as increasing the release of iron and copper oxidants and, finally, increasing the activity of inducible NO synthase. In addition, there is one report of low pH increasing the activity of one of the collagenases.

E. Near-IR spectroscopic signature of hydoxyethyl starches and its application in detecting "leaking" plaque

In an effort to determine which of the many small plaques (that confer ≤50% diameter stenosis) are indeed dangerous, we hypothesized that angiography ought to be able to detect two potential risk factors: 1) fissuring of the cap, and 2) leaky plaque microvessels (due to enhanced permeability during angiogenesis, Fig. 43) We identified plaques with "blush" (faint mural contrast remaining after package of the angiographic contrast dye column). As shown in Fig. 44 (a,b,c,d), blush was seen in only 30% to 50% of plaques that subsequently progressed, but almost all plaques with blush progressed within months. In an alternative method, a suitable near-IR indicator compound could also be used as a tracer. The presence of the indicator compound in the interior of a plaque provides a marker that plaque is "leaking" by detecting its characteristic near-IR reflectance spectrum. There are a number of suitable near-IR absorbing materials which can be taken up and sequestered by a leaky plaque. Alternatively, extravasation of vasa vasorum could be used, such as a biocompatible compound that has a distinctive near-IR absorbance spectrum easily distinguishable from the spectra of other plaque-related analytes. For example, the hydroxyethyl starches HESPAN™ and PENTASPAN™ and dextran are detectable by near-IR spectroscopy. These biocompatible starch-based macromolecules are being investigated for use as a plasma replacement or extender during routine plasma exchange[188-189], and have also been

PLAQUE upside-down
(Normal Saline)



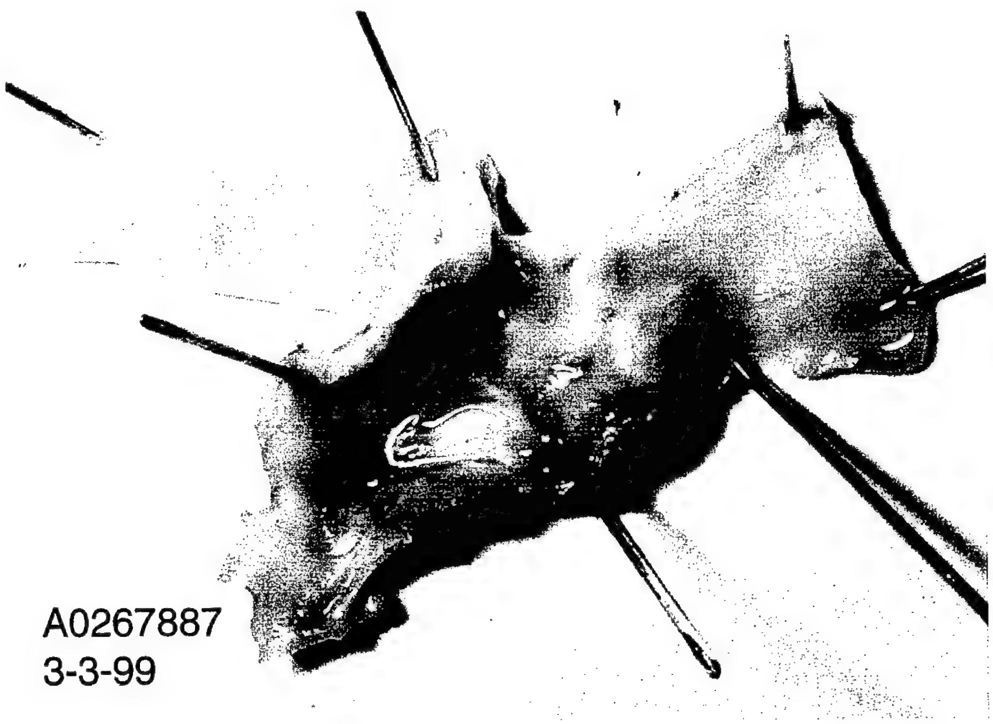


FIG. 41

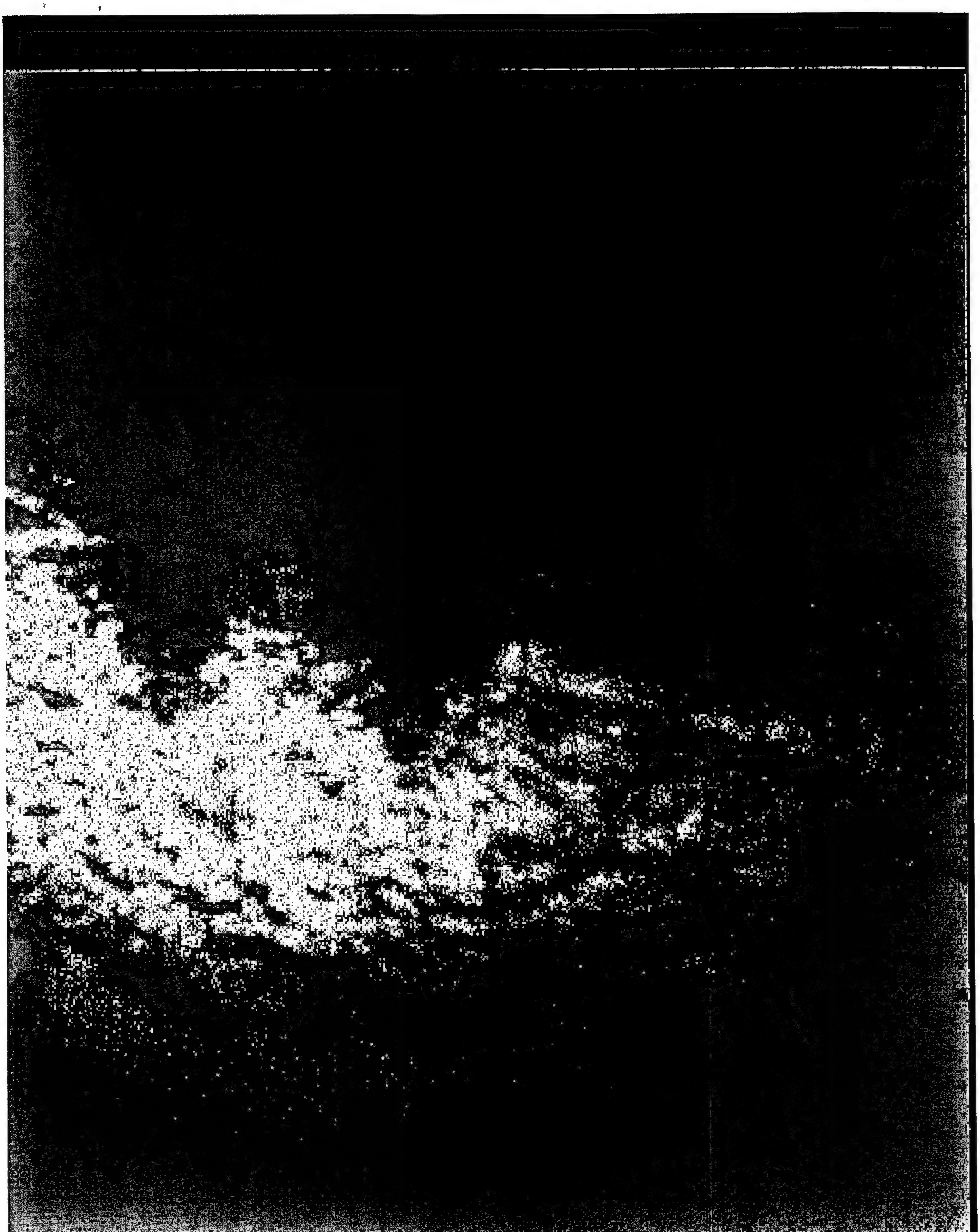


FIG. 42

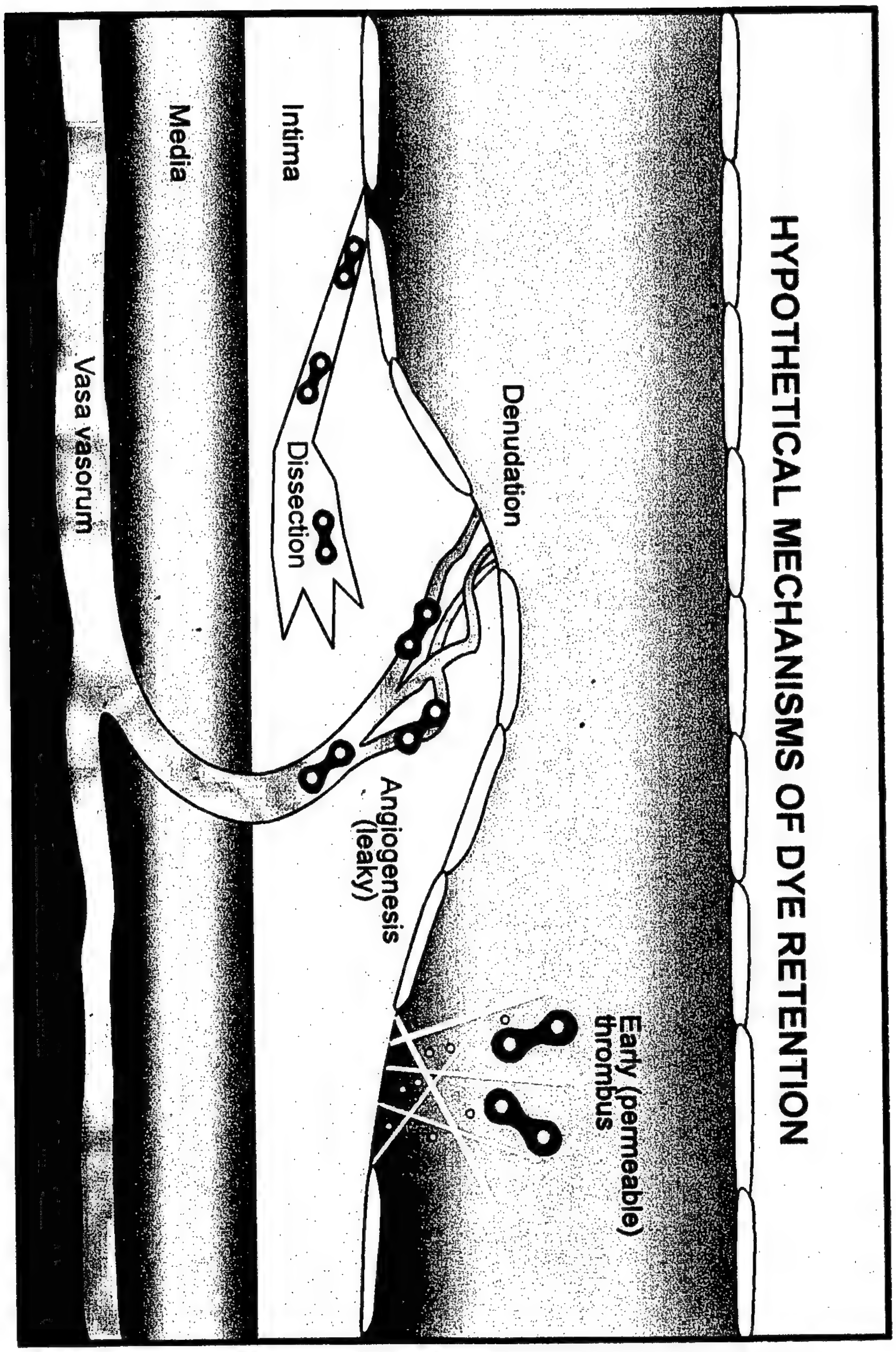

HYPOTHETICAL MECHANISMS OF DYE RETENTION
Intima
Media
Vasa vasorum
Denudation
Dissection
Angiogenesis (leaky)
Early (permeable) thrombus

FIG. 43

Figure 44 c) Frame from 44a) taken 2 frames later than in 44 b). Dye retention is more clearly seen in the proximal RCA segment as dye has continued to clear from neighboring segments.

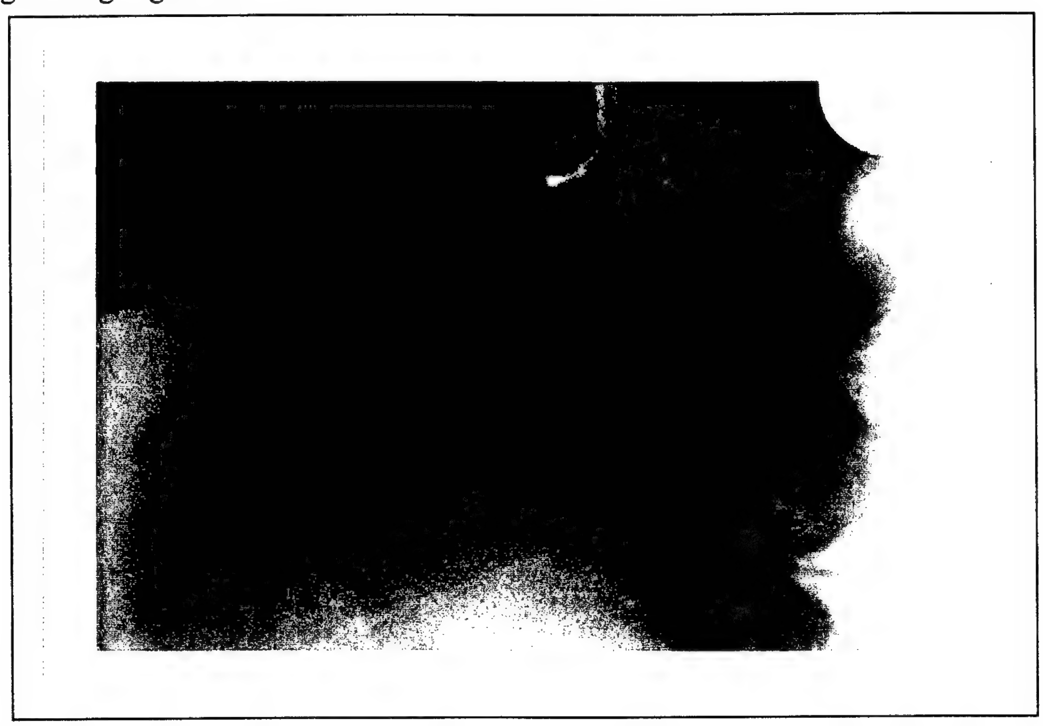


Figure 44 d) Follow-up angiogram of 44a) taken 61/2 months later. The artery is occluded at the side of earlier dye retention.

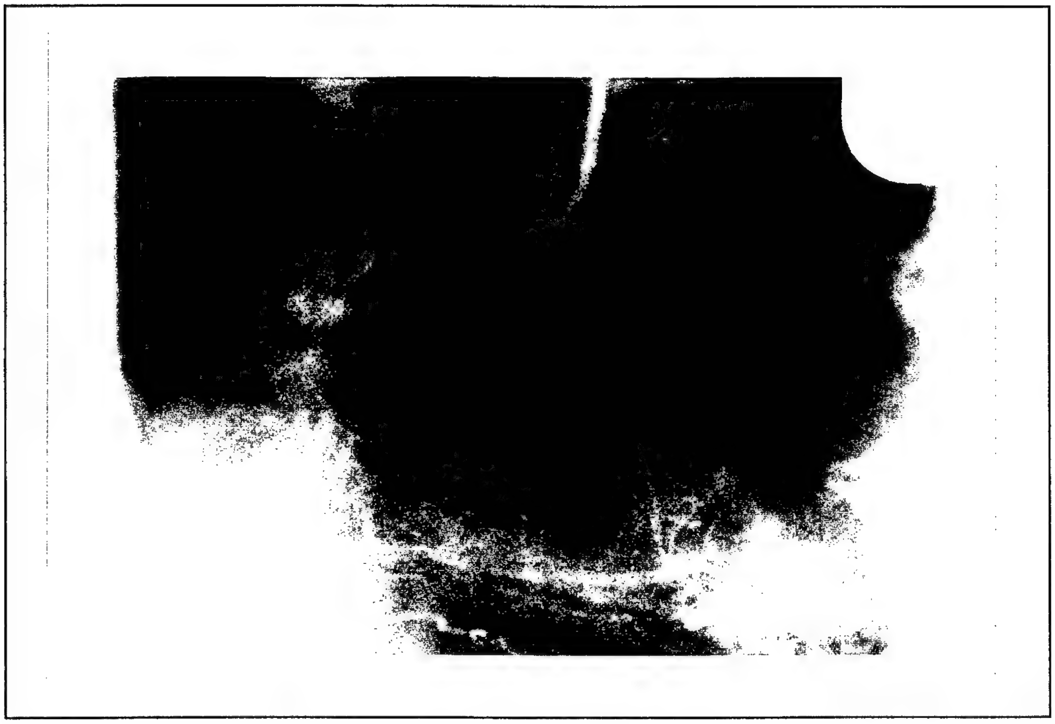


Figure 44 a) Right coronary angiogram (Left anterior oblique projection) showing several border irregularities in the artery's proximal segment.

Figure 44 b) Frame from 44 a) taken late in the washout phase. Dye retention is noted along the outside curve of the proximal RCA segment.

employed in detecting pulmonary leakage in patients with acute lung injury and acute respiratory distress syndrome (J. Wang, PhD. Dissertation 1998).

In an attempt to explore this possibility in a phantom, human carotid endathectomized atherosclerotic plaque were collected and the near-IR spectra were obtained immediately after injection of 0.1 ml Hetastarch (6% in 0.9% NaCl) into the plaque. Three different spectra are shown in Fig. 45. The top portion is the spectrum of Hetastarch (6% in 0.9% NaCl injection, Abbott Laboratories, North Chicago, IL). The middle portion is the spectrum of plaque. The bottom portion is the spectrum taken after Hetastarch was injected into the plaque. The second derivative spectra clearly show the spectra changes after injection of the Hetastarch. Hetastarch is an artificial colloid pharmacologically classified as a plasma volume expander. It is regularly used in the clinic and is safe. We propose to diagnose the plaque "leakage" using near-IR spectroscopy in combination with this biocompatible compound that is very likely to be retained after intracoronary injection. In fact, it can easily locate dangerous lesions with intraplaque hemorrhage.

Near-IR spectroscopic correlation of other potential indicators of functional status of atherosclerotic plaque

We hypothesize that the concentrations of oxygen, reactive oxygen species (including LDL cholesterol), glucose, lactate, and nitric oxide derivatives are likely more variable in vulnerable plaques than in less active (stable) plaque. Although hypoxia in atherosclerotic lesions was previously reported it is not known whether this alters the near-IR spectrum of plaque.

SUMMARY

We have discovered 1) that plaques vary markedly in pH 2) that leaky plaques are prone to progression and 3) vulnerable plaques have unique near-IR spectral features.

In further work proposed in our FY '99 BAA application (under separate cover) we propose to extend these findings to:

1) determine the contributions of NO, pH, O_2, reactive oxygen species and glucose to the near-IR "signature" of vulnerable plaque.
2) develop NIR and IR spectroscopy imaging catheters for detection of vulnerable plaques and eventually for detection of other inflammatory foci including abscesses and tumors.

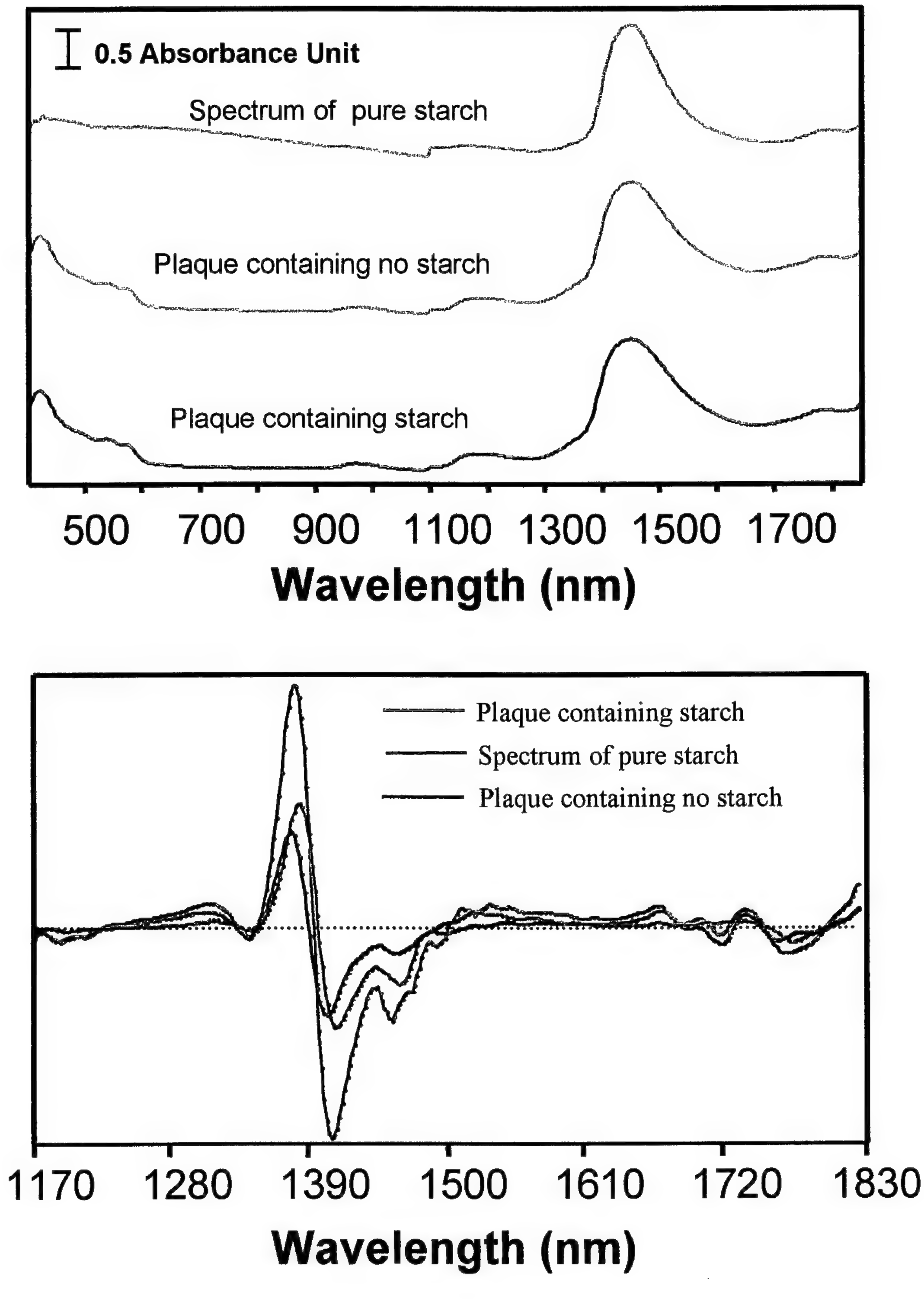


Fig. 45 Upper trace is near infrared spectra of atheroslerotic plaque, containing starch, containing no starchand and pure Hetastarch® in spectroscopic region between 400-1850 nm. Lower trace is the second derivative of these spectra demonstrate differences in the NIR region between 1170-1830 nm.

Hypothesis for pH & Plaque Rupture

Chlamydia, HSV, H pylori
cytokines

activated ⇑ → (+) **low pH** → reduced antioxidant defense ⇑ (cystein and histidine)
macrophages → release metal oxidants ⇑ (Fe and Cu)
(**heat production** ⇑) → increase iNOS activity ⇑

low Glu
high lactate
low O2
(anaerobic status)

Vicious Cycle ?

Ox-LDL ⇑ ← oxidative ⇑ stress ←

□

Acidic pH

□ ?

activate plaque acidic proteases

□

plaque softening

□

plaque rupture

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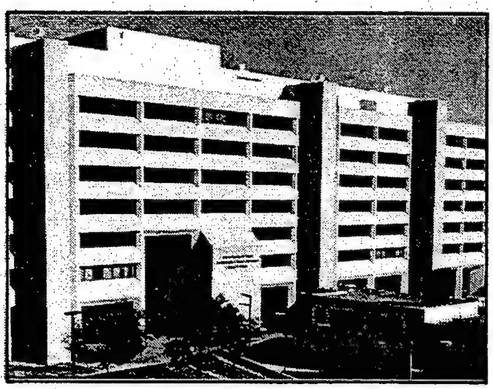
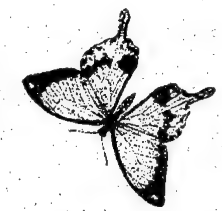
Personnel Partially Funded by DREAMS/U.S. Army Grant From Inception, 11/1/97, through 10/31/98

LAST NAME	FIRST NAME, MI
Amirian	James H.
Barham	Barbara
Berridge	Brian
Bi	Weizhen
Bigley	Joyce
Boerwinkle	Eric
Bosley	Jennifer
Byler	Denise
Casscells	S. Ward
Chang	Jiang
Chen	Yu-Fang
Cid	Emma
Cochran	Barbara
Conger	Jeff
Costas	Gil
Cui	Xiaoming
DiMauro	Jeannie
Doyle	William M.
Engler	David
Felli	Patricia R.
Groefte	Merete
Hamid	Randolph
Hassan	Khaled
He	Dacheng
Inman	Rex
Kamitani	Taeko Fukuda
Kincaid	Johnna
Kito	Katsumi
Lal	Birendra N.
Leonard	Shelly
Li	Bing
Li	Miao
Li	Tz-Rung
Li	Xiaole
Lodato	Robert F.
Malik	Basit A.
McKay	Kathleen
Mendez	Reinaldo
Morrison	Alanna
Naghavi	Morteza
Nguyen	Hung P.
Pai	Shobha V.
Pan	Su
Parks Jr	Carnel
Pasceri	Vincenci
Radovancevic	Branislav
Rosenstrauch	Doreen
Savoy	Michael
Scott-Burden	Timothy
Strobel	Henry W.
Tamez	Danny
Wada	Hiroyoshi
Wang	Jing
Willerson	James T.
Wu	Xiaobing
Wu	Henry D.
Yadav	Maneesha
Yeh	Edward T.
Zoldhelyi	Pierre

APPENDIX



Scoop



Friday, March 26, 1999

THE UNIVERSITY OF TEXAS - HOUSTON MEDICAL SCHOOL

EVENTS TO KNOW:

- **Pediatric Review and Update 1999, TOMORROW, Sat., March 27**, 8 a.m. - 4:30 p.m., MSB 3.001. Call 713-500-5249.
- **UT-Houston Honors Convocation**, Tues., **March 30**, 4-5:30 p.m., SPH auditorium.
- **ERC's Easter Basket Drawing**, Tues., **March 30**, 12:30 p.m., Leather Lounge.
- **Kirkendall Endowed Lectureship, Dr. Nancy Dickey**, President, AMA, Med. Schl. grad, Tues., **March 30**, 1-2 p.m., MSB 3.001.
- **Classified Staff Workshop**, Wed., **March 31**, 7 a.m.-4:30 p.m., Sheraton Astrodome, 8686 Kirby Drive.
- **Pediatric Hypertension Conference**, Thurs-Sat., **April 15-17**, Woodlands Conference Center, contact CME, 713-500-5249.

Note - Effective **May 1**, your I.D. badge will be needed for building entry weekdays 6 p.m. to 6 a.m. and on weekends. See *Scoop* next week for further details on your I.D. badge and its use.

UPDATE - A packed, standing-room-only crowd attended **Dr. C. Everett Koop's** Medical School lecture last week in 3.001. The former Surgeon General was both entertaining and visionary as he delivered insights on "Ten Critical Health Issues For the 21st Century."

UTmost Interest

Dr. Stanford M. Goldman, Professor and Chairman of Radiology, was elected Secretary-Treasurer of the American Society of Radiology at the 10th Annual Meeting held in Las Vegas, March 10-14, 1999.

Hot News

CHILDHOOD CANCER BENEFIT

Monday, **March 29**, 7:15 p.m., the Edwin Hornberger Conference Center's walls, at 2151 W. Holcombe, will resound with the sounds of the Fifties, "At the Hop," a gala for families dealing with childhood cancer. The fundraiser is open to the public. For information, call Candlelighters, a family alliance, 713-270-4700.

FACULTY AFFAIRS ANNOUNCES JUMP START RECIPIENTS

The Office of Faculty Affairs (OFA) announced their 1999 Jump Start Program recipients as **Dr. Rebecca Girardet**, Pediatrics; **Dr. Carrie G. Markgraf**, Neurosurgery; **Dr. Joan M. Mastrobattista**, Obstetrics, Gynecology and Reproductive Sciences; **Dr. Eugenia Mileykovskaya**, Biochemistry and Molecular Biology; **Dr. Linda Z. Nieman**, Family Practice and Community Medicine; and **Dr. Brian R. Pike**, Neurosurgery. Jump Start provides funds for short-term support (2 - 6 weeks) allowing faculty members to obtain additional training or experience needed to begin new research projects or to redirect ongoing studies. The Jump Start Program is in its third year and helps faculty to broaden their research experience, adding knowledge and expertise to their projects, both locally and through travel to specific research sites.

Graduation Activities, June 4 and 5

__Senior Awards Ceremony & Dean's Reception__ - The Senior Awards Ceremony will be held on Friday, **June 4**, 6 p.m. at the Edwin Hornberger Conference Center, followed by the Dean's Reception. Graduates, their families and friends, and faculty are cordially invited to attend all events. For more information, contact **Pat Caver**, 500-5170.

__Graduation__ - On Saturday, **June 5**, 10 a.m., graduation will take place at the George R. Brown Convention Center. **Dr. Phil H. Berry Jr.**, Texas Medical Association president in 1997-98, and organ donor recipient, will be the graduation speaker. Dr. Berry has written numerous articles, including "A Call to Physicians - Live and Then Give" and "Ethics in Transplantation." He is a member of numerous organizations including the United Network of Organ Sharing, the Flying Physicians' Association, and is a Freedoms Foundation Award recipient.

Dr. Phil Berry

THE PROBLEM OF SUDDEN CARDIAC ARREST...

To conquer cardiac arrest, the American Heart Association has recommended the wider application of automatic external defibrillators (AEDs). These electronic devices "could save many thousands of lives a year in Texas," said Director, Division of Cardiology, **Dr. Ward Cassels**. Both Casscells and **Dr. Red Duke**, Surgery, have been sharing insights with Texas lawmakers on the importance of legislation that would employ these life-saving tools more widely. The bill has now passed the Texas House and the key Senate committee. Every year, sudden cardiac arrest strikes down 350,000 people. Often seconds stand between the victim and life or death. Studies have shown that the survival rate of these victims is dramatically higher in forward-thinking cities with quick Emergency Medical Services (EMS) response times. Drs. Casscells, Duke and others are linking advanced medical tools and lives saved.

Dr. Ward Casscells

THE UNIVERSITY OF TEXAS-HOUSTON
HEALTH SCIENCE CENTER
Medical School

L. Maximilian Buja, M.D., Dean
Bryant Boutwell, Dr.P.H., Assistant Dean
Colleen O'Brien, Editor
e-mail: cobrien@dean.med.uth.tmc.edu
FAX: (713) 500-0597
E-Scoop online: http://www.med.uth.tmc.edu
Produced weekly by the Office of Community Affairs and Public Education

ANIMAL CARE PROGRAM HERE RECEIVES HIGH MARKS

The Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) gave high marks to **Dr. Bradford Goodwin**, Executive Director, and his associates, Center for Laboratory Animal Medicine and Care, in their March 12 full accreditation citation. Specifically, the AAALAC commended them for "the outstanding varied and comprehensive surgical program, directed by **Dr. Chris Smith**, Animal Laboratory Medicine, and staff. Also noteworthy were the high level of sanitation, the excellent administrative support for the program, and excellent veterinary care provided for this complex and diverse animal care and use program." (See *Scoop* Dec. 11, 1998 for earlier report). Congratulations!

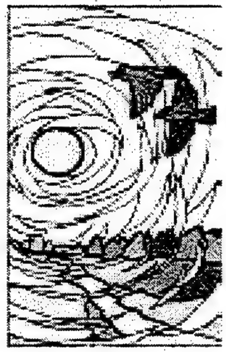
MATCH DAY - A GOOD DAY

Match Day enthusiasts, UT-Houston Medical School style, last Thursday, March 18. From left, Gloria Maguadog, Christina Cano-Gonzales, Stacey Muhammad, Michelle Torres, Jose Torres, and Marco Garza.

UT-Houston Medical School has 195 graduating seniors, and of those 108 matched with residency programs in Texas and 57 matched to UT-H programs. Also, 60% got their first choice, 20%, second choice, and 9%, third choice. Among specialty choices: 33 seniors will be doing an internal medicine residency; 30, family practice; and 20, pediatrics. Congratulations to all.

Nationally, 94% (13,707) of U.S. medical school seniors, who participated in the match, received a first-year residency training position. The National Residency Matching Program attributes this success to the preparedness of the seniors. The implementation of a web-based system this year provided more time for unmatched applicants to locate and secure a position.

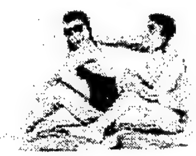
"All but two of our programs filled immediately through the match," commented **Dr. Patricia Butler**, Associate Dean for Educational Programs. At UT-H, there are 155 new residency slots among 18 programs. "Also, our residency program directors I have spoken with are pleased with the quality of the residents they recruited," added Butler. Two orientations for the incoming residents will take place on **June 23** and **30**.



AVOID THAT SUNBURN

It's that time of year. As the days get longer and Spring Break wraps up, don't forget to take precaution from sun exposure. Take time to review a few sun safe tips that can reduce you and your family's risks for skin cancer. "Since skin cancer (squamous and basal cell carcinoma) is linked to cumulative ultraviolet light exposure over your lifetime, parents should be especially attuned to their role in teaching children good sense in the sun," says **Dr. Robert Jordon**, chairman of the Department of Dermatology and a former board member of the American Academy of Dermatology. While the following sun safe tips for parents has appeared in *Scoop* in previous years, they are worth repeating.

- Keep small infants and tots under one year old out of direct sunlight. Newborns should be protected from the sun at all times. Youngsters who are fair-skinned, with blue eyes and blond or red hair need extra protection and are at highest risk.
- Apply a sunscreen with an SPF of at least 15 on all exposed areas at least 20 minutes before going outdoors...not just before going to the beach. Apply liberally and re-apply frequently, paying special attention to the face, neck, ears, upper chest, arms and legs. Consider using a sunblock such as zinc ointment on the nose if your child's nose tends to burn easily. Use a waterproof or water-resistant sunscreen on youngsters who swim.



- Limit sun exposure during peak hours, 10 a.m. – 2 p.m. Teach the "Shadow Test" to your children. If your shadow is shorter than you are, get out of the sun.
- Cover up your child with a sun hat, long-sleeved shirt and long pants for better protection.
- Remember that about 80 percent of the sun's rays penetrate the clouds. Sunscreen is needed even on cloudy or hazy days.
- A tan doesn't mean that your child is protected. Keep using a sunscreen even on a tanned child.
- If your child is taking medication, check with your doctor before allowing him/her out in the sun as certain medications can react to sunlight, causing an itchy rash, redness or swelling.
- Examine your child's skin regularly. Watch for any new raised growths, sores that don't heal, changes in moles or any new moles or itchy patches. While skin cancer is rare in youngsters, it is not unheard of in teenagers.
- Set a good example for your children by using sunscreens yourself.

NOT THE SAME OLD SONG & DANCE

Healthcare and the Arts Series '99: Discovering Self Through Healing and the Arts continues through April, noon - 1 p.m., MSB 2.006. Remaining dates and topics:

- Monday, **March 29, Dr. John Leinhard**, "I Engage and Then I See, Part I"
- Wednesday, **March 31, Dr. John Leinhard**, "I Engage and Then I See, Part II"
- Monday, **April 5, Dr. Alan Blum**, "Drawing Patients Toward Our True Selves"
- Wednesday, **April 7, Lakshmi Vishwananathan**, "Journey of Self – Discovery Through Dance"
- Monday, **April 19, Kirk Farris and Dottie Price**, "Songwriting: A Leap of Faith, Discovery for Yourself and Others."

John Everett / Chronicle

Sherri Luehr-Kirk, left, shows Betty Yoder how the automated external defibrillator works. Yoder's life was saved by use of an AED when her heart failed.

Taken to heart

Houston businesses consider defibrillators

By LESLIE SOWERS 376
Houston Chronicle

Last spring, while planting roses at her Memorial-area home, Betty Yoder began to feel different than she had ever felt before. And she did not feel well.

Concerned about the chest pain, she went inside and called 911. She remembers telling the operator that her back door was unlocked. The next thing she remembers was being asked to blink her eyes to indicate she could hear.

Her heart had stopped. Rescue personnel had used an automated external defibrillator to shock it back to a regular beat.

Yoder was lucky on two counts. Emergency help arrived promptly. And they were equipped with the AED.

"Without the AED, I wouldn't be here," Yoder, 54, said Tuesday in an American Heart Association workshop introducing this device to Houston business leaders. In Houston, the Heart Association has a campaign to encourage corporations to acquire AEDs and train designated employees in their use.

The most common locations for sudden cardiac arrest are the home and large public facilities, including the

See HEART on Page 3D.

second Page of Heart

Heart

Continued from Page 1D.

corporate environment. Each day about 1,000 Americans suffer cardiac arrest. More than 95 percent of them die, often because defibrillators are not available in time, according to the Heart Association.

Training more people to administer CPR has brought the survival rate from one in 100 to one in four, and the Houston chapter of the Heart Association hopes that training more people to use the AED will save even more lives – perhaps as many as 20,000 per year.

"In a sudden collapse, time is of the essence," said David Persse, City of Houston Emergency Medical Services director. "Often in a cardiac arrest the heart goes into a twitching rhythm, and the definitive treatment is shock."

While Persse said the city's EMS response time is excellent, circumstances sometimes cause unavoidable delays. Traffic presents one obstacle. And waiting for an elevator in a downtown office building can add life-threatening minutes. An AED at the job site might make a life-and-death difference.

If corporations purchase AEDs, does this mean the department secretary is going to be shouting, "Clear" and shocking the CEO to the ceiling?

AED reality is much less dramatic than the high drama of the television emergency room. No medical knowledge is necessary to use the battery-powered device, which directs the process by clear audio instructions and visual display.

Trained personnel need only attach two electrodes in adhesive pads to the victim's chest, stand clear and wait for the device to analyze the heartbeat. If, and only if, shock is needed, the user is instructed to push one button. Error is unlikely since the machine will not deliver an unneeded shock. Helpers are instructed when to avoid contact with the victim to avoid shock themselves.

In some instances, the machine will recognize that the heart does not need shock, yet there is no pulse. In these situations, responders are told to administer CPR until help arrives.

The Heart Association recommends that the device be kept in a central location accessible to the employee trained in its use. The goal is to ensure that every building has at least one of the devices. Currently, they are on hand at Hobby and Bush airports, the Memorial Park track, the Houston Fire Department, the mayor's office and at the Astrodome. Health professionals also have them on site at all big public events.

Two possible barriers to broader availability of AEDs are cost and liability issues.

Four manufacturers supply AEDs, and their cost averages about $3,500 to $4,000. Since the devices first became available in the 1980s, they have become smaller and simpler to use and maintain. An industry representative said the technology improves constantly and price continues to decrease.

Attorney Lee Shuchart said a "good Samaritan" law in Texas and a number of other states offers broad protection to citizens who try to help in a crisis. She said a number of jurisdictions, including Texas, may pass laws that would offer immunity to those trained in using the device. This would not affect the manufacturer's liability for the safety of the device.

Shuchart said that only a few lawsuits have been filed against good Samaritans in Texas, and that both state and national courts have favored broad protection of those who try to help. She said, in fact, that one airline was found negligent in failing to provide an AED, which would have prevented a death. As a result, several airlines have responded by making AEDs available on all flights.

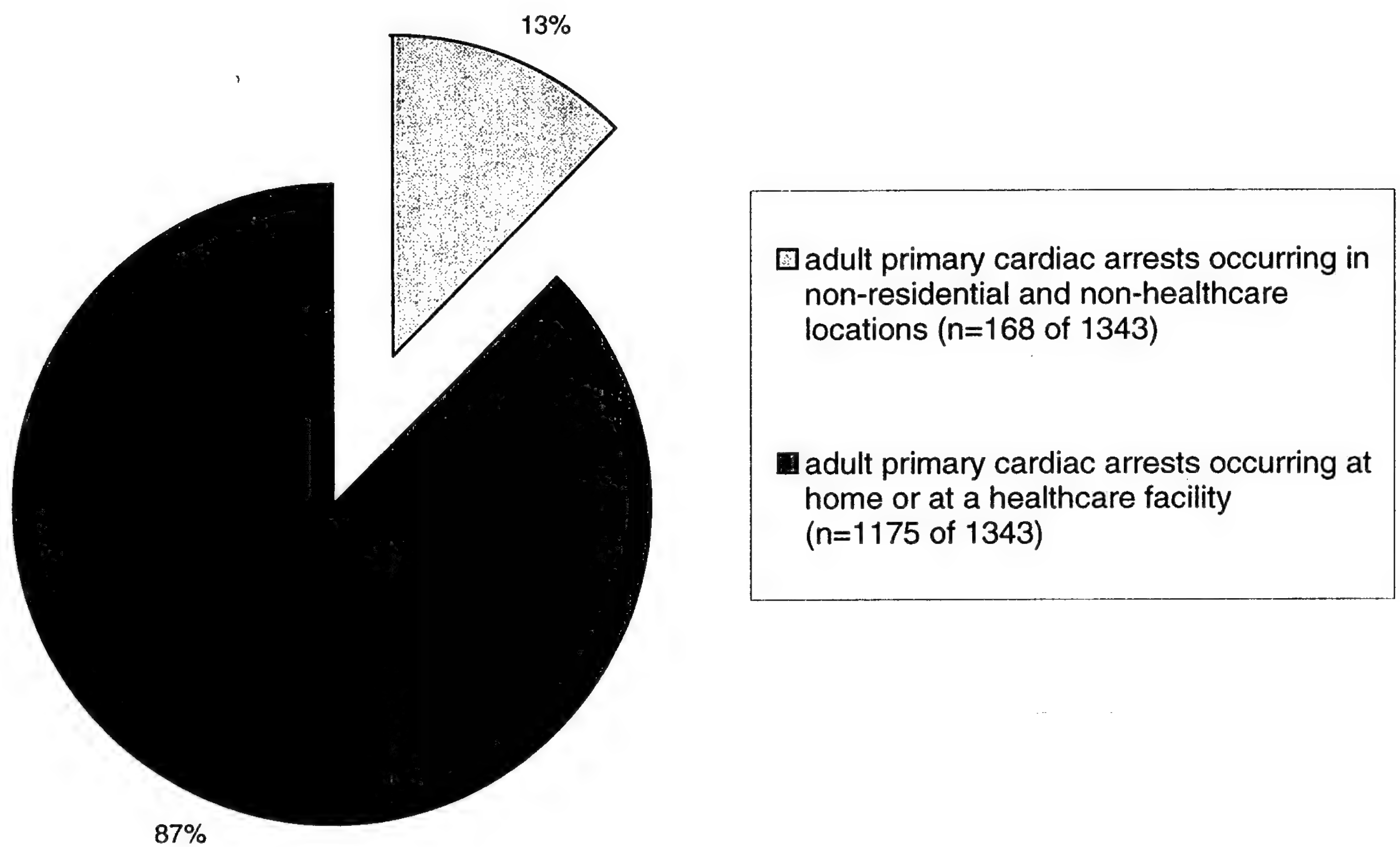
Houston is among the cities seeking to increase the use of AEDs, and the only city to sponsor a corporate awareness campaign. Training in the use of the device may be provided by the manufacturer, or the Heart Association can provide a trainer for a nominal fee. Interested business leaders can call the American Heart Association at 713-610-5000 to get information about AEDs and training. Some states are providing AEDs for communities unable to afford them, and at the national level, the Heart Association has some funds available to subsidize AED purchase.

After Yoder was revived by the AED, she spent two weeks in the hospital for testing. She said the results were inconclusive, but she did have an internal defibrillator implanted in case such a heart spasm occurs again.

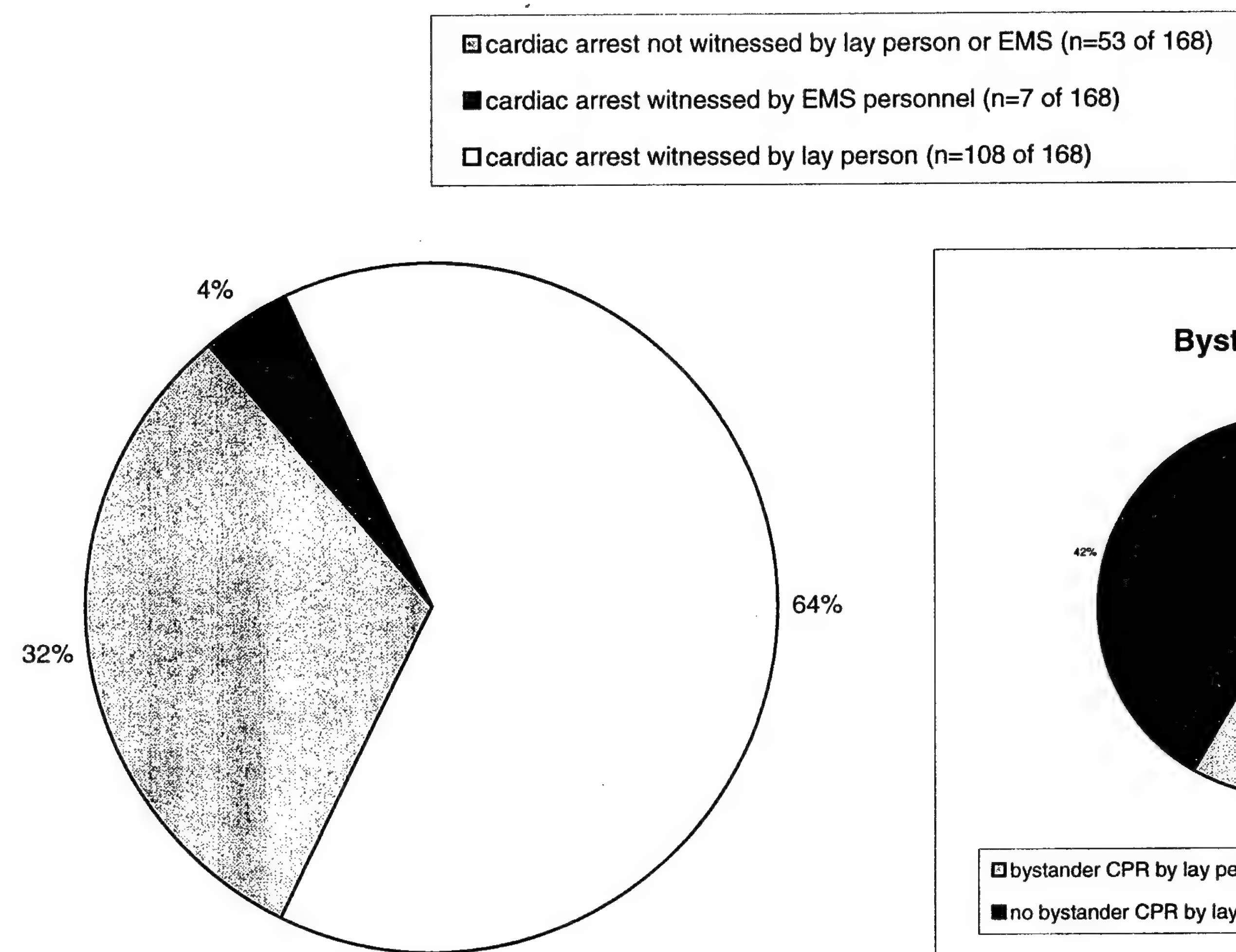
She said she takes her health a little more seriously, especially since she realized how her death would affect her husband and two grown children.

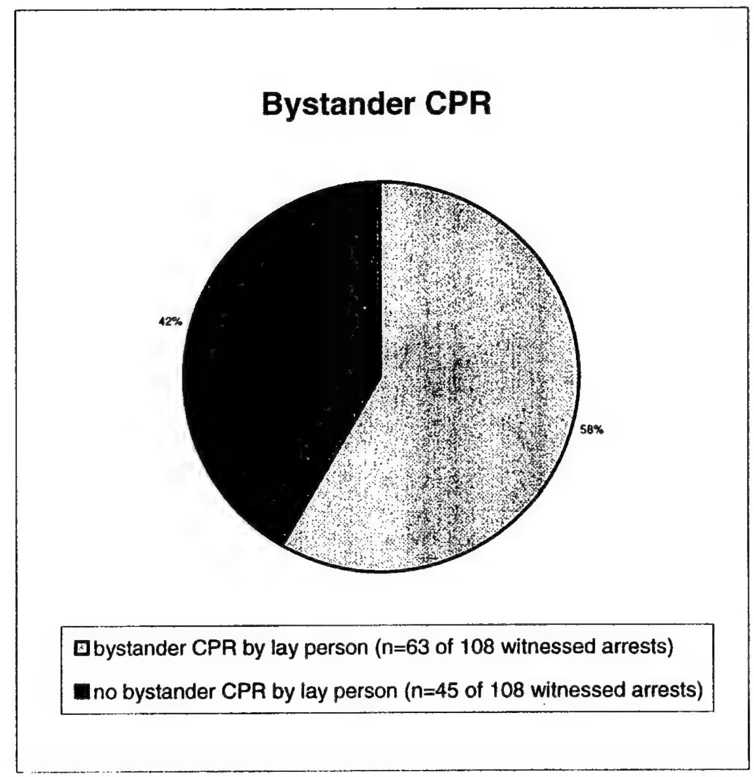
"I have become a cheerleader for the AED," Yoder said. "And so has my family."

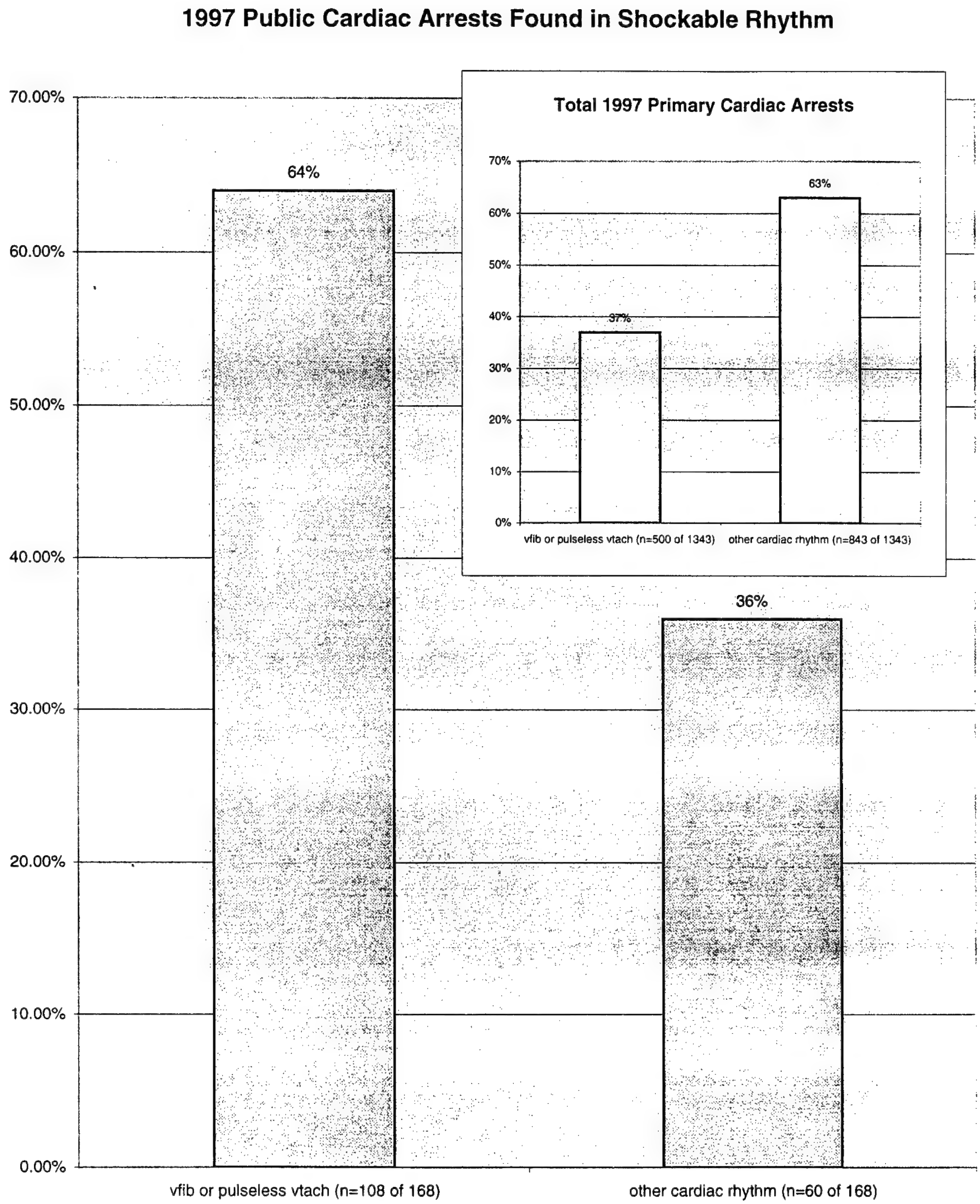
1997 Houston Public Cardiac Arrests

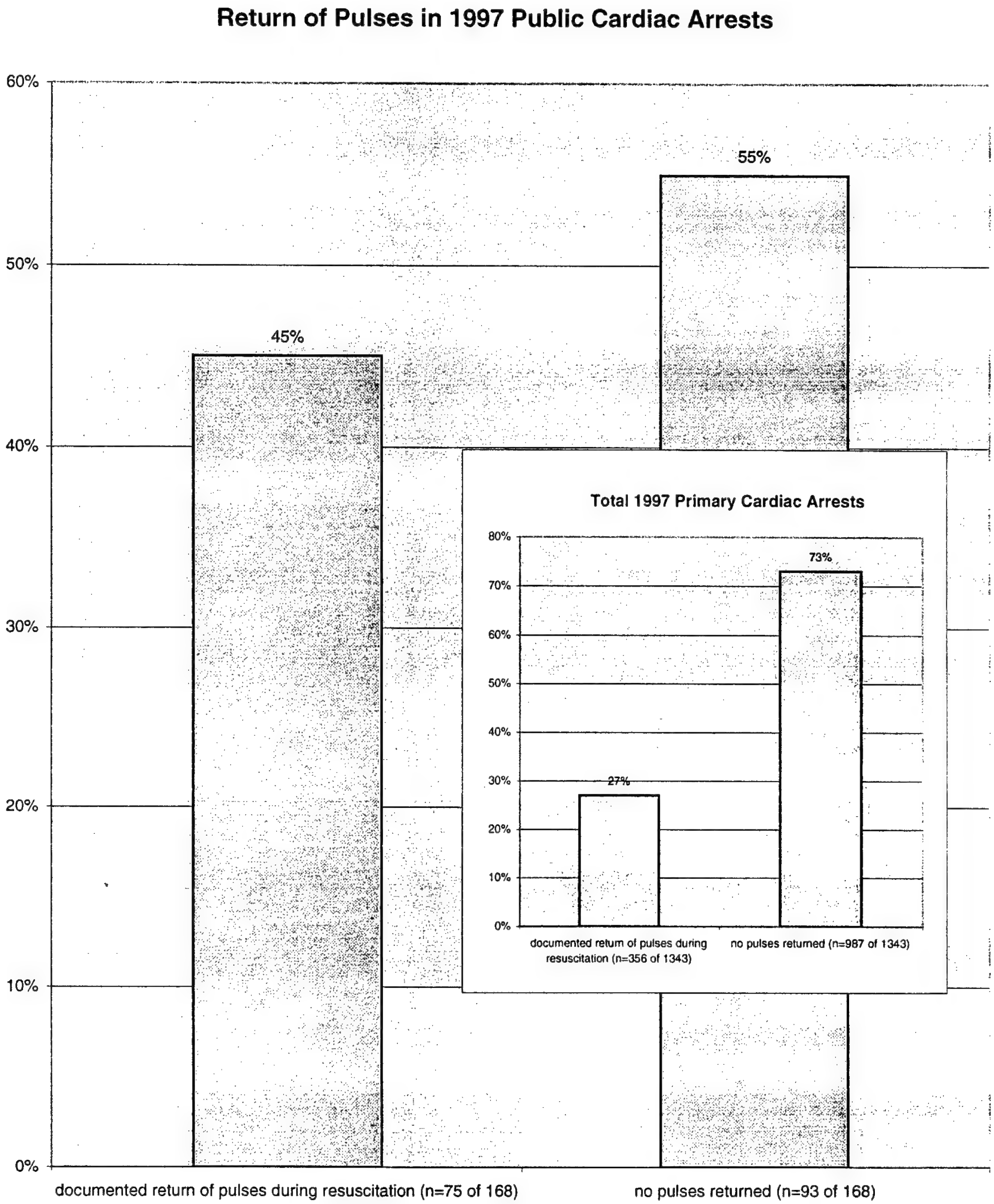


1997 Witnessed Public Cardiac Arrests

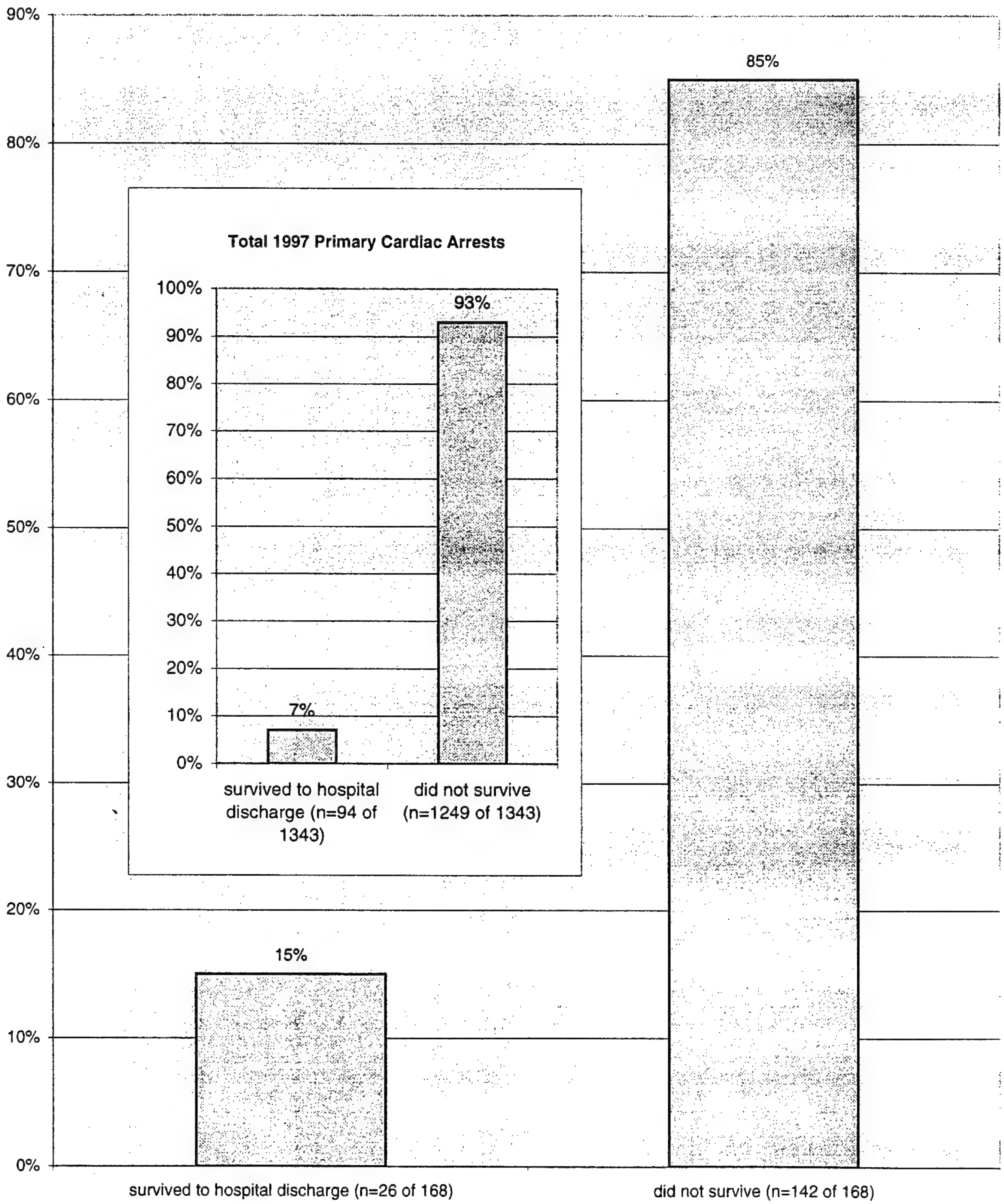




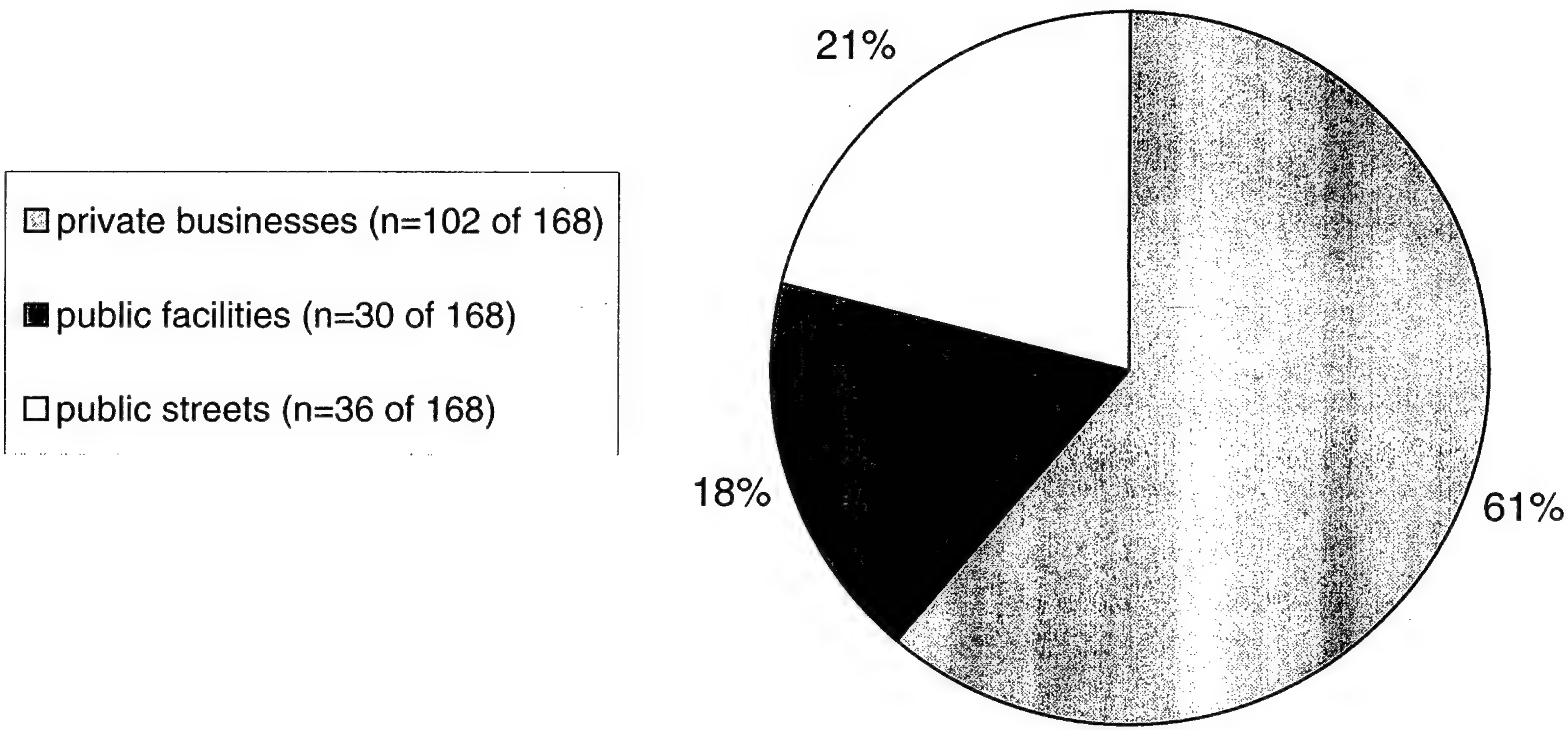

1997 Public Cardiac Arrests Found in Shockable Rhythm
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
64%
36%
vfib or pulseless vtach (n=108 of 168)
other cardiac rhythm (n=60 of 168)
Total 1997 Primary Cardiac Arrests
70%
60%
50%
40%
30%
20%
10%
0%
37%
63%
vfib or pulseless vtach (n=500 of 1343)
other cardiac rhythm (n=843 of 1343)


Return of Pulses in 1997 Public Cardiac Arrests
60%
50%
40%
30%
20%
10%
0%
45%
55%
documented return of pulses during resuscitation (n=75 of 168)
no pulses returned (n=93 of 168)
Total 1997 Primary Cardiac Arrests
80%
70%
60%
50%
40%
30%
20%
10%
0%
27%
73%
documented return of pulses during resuscitation (n=356 of 1343)
no pulses returned (n=987 of 1343)

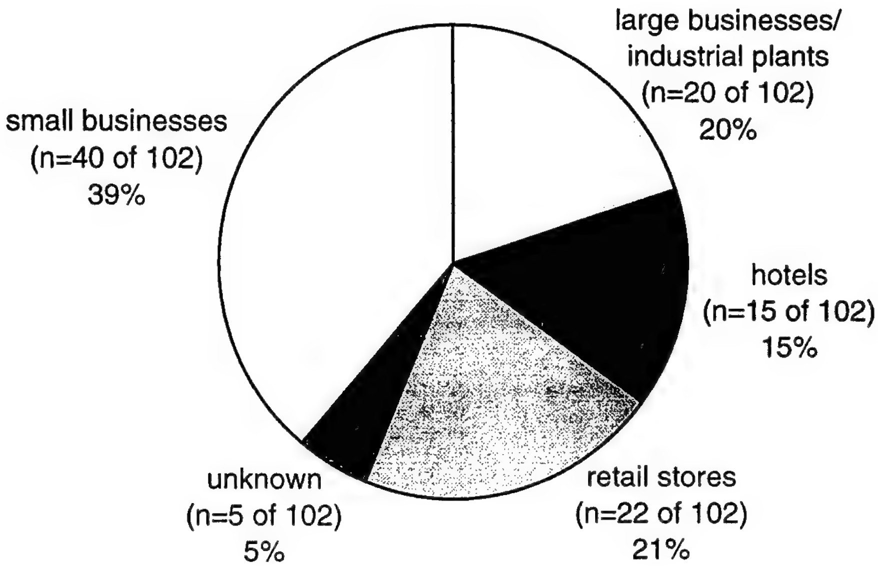
Survival of 1997 Public Cardiac Arrest Patients



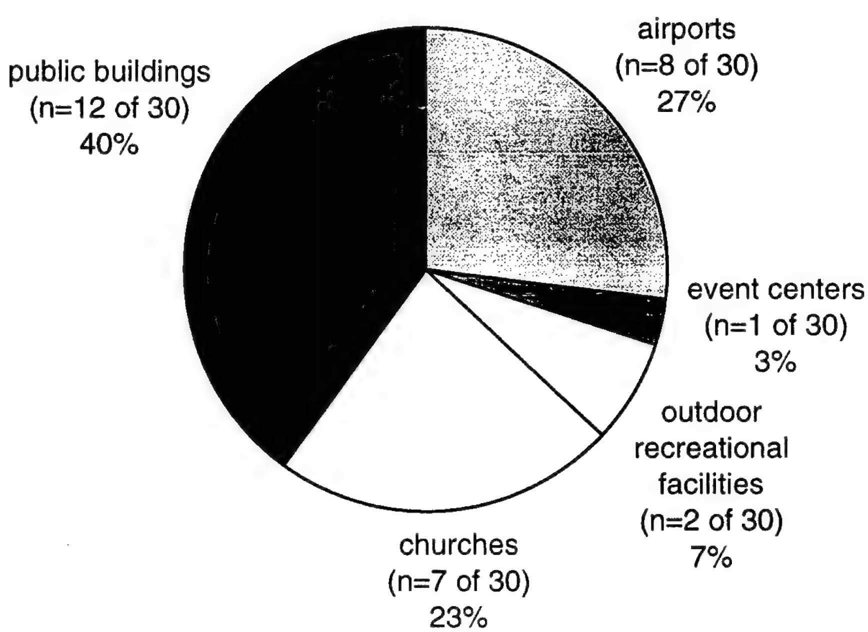
Locations of 1997 Adult Public Cardiac Arrests



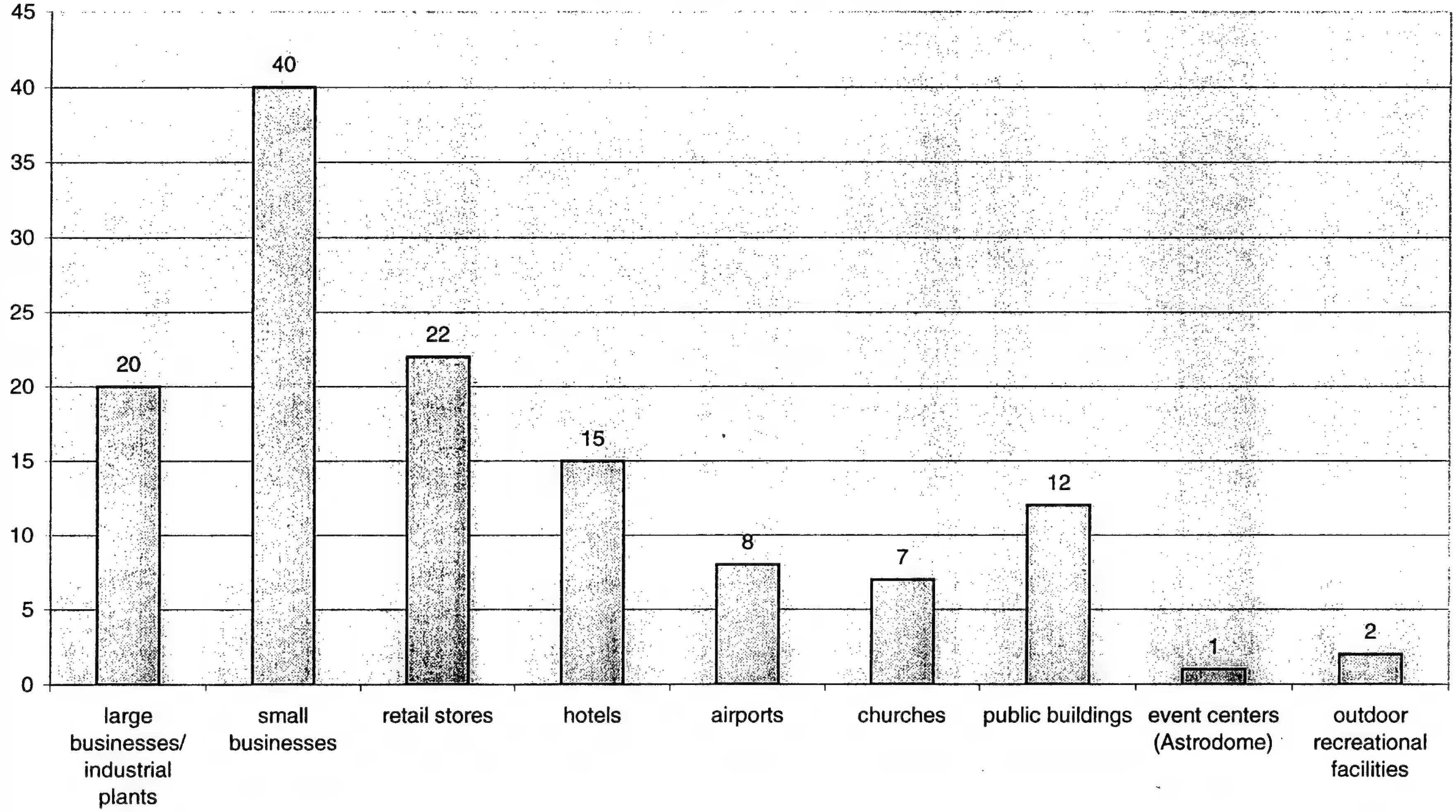
1997 Adult Cardiac Arrests Occurring in Private Businesses



1997 Adult Cardiac Arrests Occurring in Public Facilities



Number of Primary Cardiac Arrests Occurring in Private Businesses and Public Facilities in 1997



1997 Public Cardiac Arrests in Houston

All adult primary cardiac arrests occurring in Houston during 1997	1343	
All adult primary cardiac arrests occurring in non-residential and non-healthcare locations	168	13%

Time of 911 call

06:00-12:00 morning	50	30%
12:00-18:00 afternoon	77	46%
18:00-24:00 evening	32	19%
00:00-06:00 night	9	5%

Age of patient

15-25 years old	1	<1%
25-34 years old	3	2%
35-44 years old	18	11%
45-54 years old	61	37%
55-64 years old	39	24%
65-74 years old	28	17%
75-84 years old	11	7%
>85 years old	3	2%

Gender

Male	137	82%
Female	31	18%

Witnessed arrest

Cardiac arrest witnessed by lay person	108	64% *
Not witnessed by lay person	53	31%
Witnessed by EMS personnel	7	4%

* Of total adult primary cardiac arrests (1343), 41% were witnessed.

L. Fletcher 10/30/98
lf691342@express.bcm.tmc.edu

Bystander CPR

Lay person performed bystander CPR after witnessed arrest	63 of 108	58% *
No bystander CPR by lay person after witnessed arrest	45 of 108	42%

* Of total adult primary cardiac arrests (1343), 14% received bystander CPR by a lay person.

Arrival time

Average time from 911 call to arrival of EMS personnel	6.4 minutes

First responder application of AED

SAD protocol by first responders	93	55%
AED applied by paramedics or supervisor	75	45%

Found in shockable rhythm

Upon AED application, patient's initial cardiac rhythm was ventricular fibrillation or pulseless ventricular tachycardia	108	64% *
Patient was found in other rhythm (asystole, PEA, IVR, etc)	60	36%

* Of total adult primary cardiac arrests (1343), 37% were found in a shockable rhythm.

Return of Spontaneous Circulation

Documented return of pulses during resuscitation	75	45% *
No pulses returned	93	55%

* Of total adult primary cardiac arrests (1343), 27% had return of spontaneous circulation.

Survival

Patient survived and was discharged alive from hospital	26	15% *
Patient died before admission or did not survive to hospital discharge	142	85%

* Of total adult primary cardiac arrests (1343), 7% were discharges alive from the hospital.

L. Fletcher 10/30/98
lf691342@express.bcm.tmc.edu

1997 Public Cardiac Arrest Locations

PRIVATE BUSINESSES	**102**	**61%**
Large businesses including industrial plants and large office buildings (Shell-1, Conoco-1, El Paso Energy-1)	20	20%
Small businesses/services Gyms/exercise clubs (Bally's-2, Q Sports Club-3, YMCA-1) Restaurants Nightclubs/bars Auto service shops, car wash, beauty salon, print shop, washateria, title company	40 (6) (9) (9)	39% (6%) (9%) (9%)
Retail stores Malls and clothing stores Grocery stores Liquor stores, car dealerships	22 (4) (9)	22% (4%) (9%)
Hotels (Harvey Suites-4)	15	15%

PUBLIC FACILITIES	**30**	**18%**
Airports (IAH-5, Hobby-3)	8	27%
Churches	7	23%
Public buildings Library, post office, high schools, universities, community centers	12	40%
Event centers (Astrodome)	1	3%
Outdoor recreational facilities (Memorial park, Kingwood golf course)	2	7%

PUBLIC STREETS	**36**	**21%**
Pedestrian on side of street	16	44%
Driver or passenger in vehicle on street	20	56%

L. Fletcher 10/30/98
lf691342@express.bcm.tmc.edu

American Heart Association
Fighting Heart Disease and Stroke

AED BILL

Date: March 2, 1999

To: AED AND PUBLIC AFFAIRS COMMITTEE MEMBERS

S. Ward Casscells, M.D.
Anne Doughherty, M.D.
Carolyn Galloway, M.P.H.,M.D.
Sherri Luehr-Kirk
Jeff Towbin, M.D.
John Sweitzer
Pat Trevisanj, R.R.T., R.N., M.S.
Mark Yeoman, M.D.
Francisco Fuentes, M.D.
James Grotta, M.D.
Linda Koening
Lewis Morgenstern, M.D.
Kim Nettleton
Toni Pool, R.N.
Joseph Swafford, M.D.
Jacquie Frelow, AHA Staff
Michele Heileman, AHA Staff
Ryan Rice, AHA Staff

From: Jacquie Frelow

Re: AED Bill – FYI

TOTAL PAGES: 11

Association, the American Red Cross, or other nationally recognized association in:

(A) cardiopulmonary resuscitation; and

(B) use of the automated external defibrillator; and

(2) a licensed physician is involved in the training program to ensure compliance with the requirements of this chapter.

(b) The Texas Department of Health shall adopt rules establishing the minimum requirements for the training required by this section.

Sec. 779.003. MAINTENANCE OF AUTOMATED EXTERNAL DEFIBRILLATOR. A person who owns or leases an automated external defibrillator shall maintain and test the automated external defibrillator according to the manufacturer's guidelines.

Sec. 779.004. USING AN AUTOMATED EXTERNAL DEFIBRILLATOR. A person who provides emergency care to a person in cardiac arrest by using an automated external defibrillator shall contact the local emergency medical services provider and notify the provider of the use of an automated external defibrillator.

Sec. 779.005. NOTIFYING LOCAL EMERGENCY MEDICAL SERVICES PROVIDER. When a person acquires an automated external defibrillator, the person shall notify the local emergency medical services provider of the existence, location, and type of automated external defibrillator.

Sec. 779.006.

physician who authorizes the acquisition of an automated external defibrillator, a person who provides training in the use of an

automated external defibrillator, and the person responsible for the automated external defibrillator are not liable for civil damages for an act performed unless the act is wilfully or wantonly negligent.

Sec. 779.007. POSSESSION OF AUTOMATED EXTERNAL DEFIBRILLATORS. Each person, other than a licensed practitioner, who acquires an automated external defibrillator shall ensure that:

(1) the defibrillator has been delivered to that person by a licensed practitioner in the course of the practitioner's professional practice or upon a prescription or other order lawfully issued in the course of the practitioner's professional practice; or

(2) if the defibrillator is acquired for the purpose of sale or lease, the person shall be in conformance with the applicable requirements found in Section 483.041.

Sec. 779.008. HOSPITAL EXEMPTION. This chapter shall not apply to hospitals licensed under Chapter 241.

SECTION 2. Subsection (a), Section 74.001, Civil Practice and Remedies Code, is amended to read as follows:

(a) A person who in good faith administers emergency care, including using an automated external defibrillator, at the scene of an emergency but not in a hospital or other health care facility or means of medical transport is not liable in civil damages for an act performed during the emergency unless the act is wilfully or wantonly negligent.

SECTION 3. This Act takes effect September 1, 1999, and applies to a person who possesses an automated external

defibrillator on the effective date of this Act or acquires an automated external defibrillator on or after that date.

SECTION 4. The importance of this legislation and the crowded condition of the calendars in both houses create an emergency and an imperative public necessity that the constitutional rule requiring bills to be read on three several days in each house be suspended, and this rule is hereby suspended.

Suggested amendments to C.S.S.B. 122/H.B. 580:

ok

(1) Amend Sec. 779.004. USING AN AUTOMATED EXTERNAL DEFIBRILLATOR. A person who provides emergency care to a person in cardiac arrest by using an automated external defibrillator shall promptly notify [~~contact~~] ~~and activate~~ the local emergency medical services provider. [~~and notify the provider of the use of an automated external defibrillator~~]

ok

(2) Amend Sec. 779.006 in its entirety and substitute the following language:

Sec. 779.006. LIABILITY EXEMPTION. The prescribing physician who authorizes the acquisition of an automated external defibrillator in accordance with this chapter, a person who provides approved training in the use of an automated defibrillator in accordance with this chapter, and the person [~~responsible for~~] who acquires the automated external defibrillator and meets the requirements of this chapter are not liable for civil damages for such prescription, training or acquisition [~~an act performed~~] unless the conduct [~~act~~] is willfully or wantonly negligent.

(3) Amend SECTION 1 by adding a new Sec. 79.007 to read as follows:

Sec. 779.007. LIABILITY OF TRAINED AUTOMATED EXTERNAL DEFIBRILLATOR OPERATORS. Only those persons who have successfully completed and complied with training and all other regulations and requirements with regard to the operation of an automated external

Gene Weathersby

defibrillator as promulgated by the Texas Department of Health and who operate the automatic external defibrillator in accordance with that training and the requirements of this statute are not liable in civil damages for an act performed during the emergency unless the act is willfully and wantonly negligent.

(4) Amend H.B. 580 by striking SECTION 2 in its entirety and renumbering the subsequent SECTIONs appropriately.

By: Nelson S.B. No. 122

Substitute the following for S.B. No. 122:

By: ____________ C.S.S.B. No. 122

A BILL TO BE ENTITLED

AN ACT

relating to automated external defibrillator devices.

BE IT ENACTED BY THE LEGISLATURE OF THE STATE OF TEXAS:

SECTION 1. Subtitle B, Title 9, Health and Safety Code, is amended by adding Chapter 779 to read as follows:

CHAPTER 779. AUTOMATED EXTERNAL DEFIBRILLATORS

Sec. 779.001. DEFINITION. In this chapter, "automated external defibrillator" means a heart monitor and defibrillator that:

(1) has received approval from the United States Food and Drug Administration of its premarket notification filed under 21 U.S.C. Section 360(k), as amended;

(2) is capable of recognizing the presence or absence of ventricular fibrillation or rapid ventricular tachycardia and is capable of determining, without interpretation of cardiac rhythm by an operator, whether defibrillation should be performed; and

(3) on determining that defibrillation should be performed, automatically charges and requests delivery of an electrical impulse to an individual's heart.

Sec. 779.002. TRAINING. (a) A person who acquires an automated external defibrillator shall ensure that:

(1) each user of the automated external defibrillator receives training given or approved by the American Heart

Amendment No. ______ By:______________

Amend H.B. 580 by striking Sec. 779.002 in its entirety an inserting the following:

Sec. 779.002 TRAINING. (a) a person or entity who acquires an automated external defibrillator shall ensure that:

(1) each user of the automated external defibrillator receives minimum training as promulgated by the Texas Department of Health and based upon science developed by the American Heart Association, American Red Cross or other nationally recognized organization in:

(A) cardiopulmonary resuscitation; and

(B) use of the automated external defibrillator;

and

(2) a licensed physician is involved in the training program to ensure compliance with the requirements of this chapter.

Defibrillator at Heart of AHA Corporate Effort

The American Heart Association (AHA) is spearheading an initiative to increase awareness about the automatic external defibrillator (AED), a device used to restart a stopped heart, and the need for public access to such life-saving equipment.

Betty Yoder, 54, can speak first-hand about the benefits of an AED. Emergency personnel used the device to revive her stopped heart after she went into cardiac arrest while gardening.

Yoder was one of several panel members discussing public use of AEDs at a recent American Heart Association workshop for Houston business representatives. Panel members offering various perspectives discussed the placement of AEDs in the community, particularly in office buildings, sports venues, and airports,

(Continued on page 19)

KRISTINA VAN ARSDEL

AHA volunteer Sherri Luehr-Kirk of The Luehr Group, Inc., and Dan Rutledge, a representative for Survivalink, demonstrate the AED.

AHA...

(Continued from page 5)

where large numbers of people congregate. All Houston fire units have been equipped with AEDs since 1991. Since that time, technology has developed so that AEDs are now lighter in weight and easier to use.

"We believe that from an EMS standpoint, by utilizing the firefighters, the fire trucks, and the ambulances the way we have, we have done everything we can to get a defibrillator to the patient as soon as possible," said Dr. David Persse, City of Houston EMS medical director. "The next step is to put it in the hands of the people that are right there with the patient.

"The whole reason we are entertaining the idea that the public can operate these is that this new second generation of devices is so much easier to use with very little training required," said Dr. Persse, a participant on the panel.

In Houston, the American Heart Association has a corporate goal of at least one unit per office building, or one unit for every 1,000 employees. AEDs average between \$3,000 and \$4,000 for a basic unit, are only available by prescription and each business must have a medical director to obtain one. The American Heart Association or the manufacturer of the device can recommend a medical director for those businesses who do not have one.

Dr. Paul Sirbaugh, assistant medical director, City of Houston EMS, and director of EMS at Texas Children's Hospital, discussed the need for training a designated person on each work shift and the importance of placing the device in a centralized location in the office building. The designated operator should be re-trained every six months in both the use of the AED and in CPR (cardiopulmonary resuscitation), said Dr. Sirbaugh.

Will it be like an episode of "ER" in the board room? No. Here's how it works: the trained operator attaches the adhesive pads to the patient, turns the device on and waits for verbal instructions from a computerized voice. The AED then takes a reading of the patient's heart rhythm and, if necessary, instructs the operator to push a button which shocks the heart. The device then assesses the heart rhythm again and may proceed with a second shock. Other instructions may follow depending on the situation. It is not possible to over-shock someone because the machine determines the level of shock, if a shock is even necessary.

According to Dr. Sirbaugh, the AEDs are only recommended for people who weigh over 90 pounds because the power of the shock is too intense for a child.

The American Heart Association estimates that each year sudden cardiac death claims at least 250,000 lives in the United States. Each minute defibrillation is delayed, the chances of survival decrease by 10 percent.

AEDs are not meant to replace CPR, but act as another link in the "chain of survival" – which consists of dialing 9-1-1, early CPR, early defibrillation, and early advanced care.

A CPR mass training will take place at the Astrodome on November 1 for both adult and child/infant CPR. The AED will be demonstrated at the training. For more information or to register, call 713-225-4CPR. ■

– KRISTINA VAN ARSDEL

AMERICAN HEART ASSOCIATION
HOUSTON DIVISION

AED AND PUBLIC AFFAIRS COMMITTEE MEETING
Wednesday, February 3, 1999
12:00 P.M.

MINUTES

Attendance: Paul Sirbaugh, MD, Sherri Luehr-Kirk, David Persse, MD, John Sweitzer, Pat Trevisani, RRT, Mark Yeoman, MD
Not in Attendance: Francisco Fuentes, MD, Carolyn Galloway, MD, James Grotta, MD, Joseph Swafford, MD, Jeff Towbin, MD, Toni Pool, RN, Caroline Osmon, Kim Nettleton, Lewis Morgenstern, MD, S. Ward Casscells, MD, Anne Dougherty, MD
AHA Staff Present: Ryan Rice, Michele Heileman, Jacqueline Frelow

I. **The minutes from the October 30, 1998 meeting were approved as written.**

II. **Project Update** – The AED Campaign is progressing well. Several corporations and individuals have contacted the AHA as a follow-up to the September 29, 1998 event. To date, approximately 66 AEDs have been purchased. Jacquie' is contacting the manufacturers for information, as appropriate.

III. **Legislative Issues Update/John Sweitzer** – The AED Bill developed by State Rep. Kyle Janek was reviewed. The bill is identical to Sen. Jane Nelson's bill. There is concern regarding Sec. 779.002, Training, #2,b. The TDH is identified for establishing the minimum requirements for training. John suggested that TDH have the AHA present the Heartsaver AED for determination of course completion requirement.
- The American Red Cross wants their name to be attached to the bill. The concern is that this could ultimately develop into a very long list. The committee agreed that getting the ARC name on the bill would give more political backing, strength, and also help with creating awareness. **John will call David Reynolds with comments from the committee.**
- John also suggested that we should meet with State Rep. Patricia Gray (Galveston), Dept. of Health and Human Services.
- There is a 3rd bill out drafted by Sen. Osgood, which addresses training. 3 AEDs have been given to the Texas Legislature: 1 Senate, 1 House of Representatives and 1 to Gov. Bush. Thanks to Dan Rutledge, SurVivalink and Mark Cate, Heartstream.

IV. **EXXON, USA AED Workshop/Sherri Luehr-Kirk** – Atty. Lee Shuchart is confirmed. RSVPs to date-25. Ryan will follow-up with Leba Shallenberger regarding media. A draft of the program was reviewed. Jacquie' will forward to Leba for finalizing. We need to invite other corporations and encourage the AED manufacturers to send the information to pending/potential clients. Jacquie' will follow up with Leba regarding parking.

V. **Other Business/Discussion**

- **AED Video** – The committee needs to update the PAD video as soon as possible. Sherri will talk with Dan Rutledge about reproducing the current video; at least 12. Also, it would be great to develop a video to distribute to interested groups, cities, etc., that are interested in the program. Ryan will research media locally. Michele Heileman will contact Pat Bowser, AHA, regarding Operation Heartbeat AED video.
- Although AED is a national AHA initiative, there are states that are not participating in the effort.

VI. **Members will be contacted regarding the next meeting.**